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Title: Use of Elicitor Sets to Characterize Cellular Signal Transduction Networks

Abstract

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Frank W.R. Chaplen

Intracellular signaling cascades can no longer be viewed as linear pathways that relay and amplify information. Often, components of different pathways interact, resulting in signaling networks. The interactions of different pathways and the dynamic modulation of the activities of the components within signaling pathways can create a multitude of biological outputs. The cell appears to use these pathways as a way of integrating multiple inputs to shape a uniquely defined output. These outputs allow the cell to respond to and adapt to an ever-changing environment. Understanding how biological systems receive, process and respond to complex data inputs has important implications for the design and utilization of sensors for a variety of applications, including toxicology, pharmacology, medical diagnostics, and environmental monitoring. This study uses the elicitor sets method, which is an experimental framework designed to monitor information

flows through signal transduction pathways. The elicitor set approach has been used to derive mechanistic interpretations from the action of Phenylmethylsulfonyl Fluoride (PMSF), a serine protease inhibitor and nerve agent analog. The elicitor panel comprises of signal transduction network effectors namely forskolin, clonidine, cirazoline and H89, each of which targets the signaling pathway at known specific points. The elicitor set experiments enable compartmentalization of the cAMP signaling pathway, examining the role played by each segment and identifying possible cross-talk mechanisms. Our experiments substantiate that selection of adenyl cyclase as the reference node and 10 μM forskolin as the primary elicitor, segments the upper portion of the G-Protein Coupled Receptor (GPCR) pathway associated with the G_q and G_i proteins. Application of the secondary elicitors, 100 nM clonidine (α2-adrenergic receptor agonist), 1 μM and 100 μM cirazoline (α1-adrenergic receptor agonists), and 1 μM and 100 μM H-89 (PKA inhibitor) fortifies the decoupling, as the system is unresponsive to clonidine and cirazoline in the presence of forskolin, while continuing to respond to H-89. Exposure of the cells to 1 mM PMSF subsequent to forskolin addition restricted the quantifiable impact of PMSF to regions of the signaling pathways below adenyl cyclase. Triggering the system by use of secondary elicitors augmented the information resolution which is reinforced by the increased sensitivity of cells to 100 μM H-89 that acts at an important checkpoint below adenyl cyclase.

Use of Elicitor Sets to Characterize Cellular Signal Transduction Networks

by
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TABLE OF CONTENTS

TABLE OF CONTENTS CONTINUED

Page

TABLE OF CONTENTS CONTINUED

LIST OF FIGURES

USE OF ELICITOR SETS TO CHARACTERIZE CELLULAR SIGNAL TRANSDUCTION NETWORKS

1 INTRODUCTION

Studies of cellular systems are beginning to show the extreme complexity of the control and regulation of systemic cellular responses to external signals, either from within the organism or from external environmental sources, including conventional drug molecules. Biological signaling pathways interact with one another to form complex networks. Complexity arises from large number of components, many with isoforms that have partially overlapping functions; from the connections among components; and from the spatial relationship between components (11). Apart from the inherent complexity of signaling networks, it has become apparent that elucidation of the components and functional properties of such networks may be additionally complicated by the existence of 'fail-safe' systems, redundancies or convergent pathways (12). Additional complications arise from observations suggesting that cellular responses are dependent not only on cell type, but also on cellular state, as well as the fact that responses from a single receptor can depend on cell state. In the context of pharmacology, it has been suggested that cellular control systems are analogous to neural networks that produce an integrated response from a multiplicity of inputs and that pharmaceutical efforts should be directed at manipulation of networks rather than at targeting of individual molecules (25, 27). One of the major requirements for targeting functional networks, rather than individual molecules, is the availability

of methods for visualizing the response of a functional network in normal conditions, under abnormal conditions (i.e. a disease state), and in response to manipulations (i.e. the administration of a therapeutic compound).

1.1 Systems Biology Approach

Systems biology does not investigate individual genes or proteins rather it investigates the behavior and relationships of all of the elements in a particular biological system while it is functioning. These data can be then integrated, graphically displayed and ultimately modeled computationally. The Human Genome Project has catalyzed a new scientific approach to biology, termed discovery science (26). It has strengthened the view that biology is an informational science, provided us with powerful new high-throughput tools for systematically perturbing and monitoring biological systems and stimulated the creation of new computational models. The objective of discovery science is to define all of the elements in a system and to create a database containing that information.

Biological systems are fundamentally composed of two types of information: genes, encoding the molecular machines that execute the functions of life, and networks of regulatory interactions, specifying how genes are expressed. All of this information was thought to be hierarchical in nature: DNA → mRNA → protein → protein interactions → informational pathways → informational networks → cells → tissues or networks of cells → an organism → population → ecologies. However, there is evidence of substantial interaction between these

levels giving rise to networks. The central task of systems biology is: (a) to comprehensively gather information from each of these distinct levels for individual biological systems and (b) to integrate these data to generate predictive mathematical models of the system.

Biological information has several important features:

- It operates on multiple hierarchical levels of organization.
- It is processed in complex networks.
- These information networks are typically robust, such that a single perturbation will not usually affect them.
- There are key nodes in the network where perturbations may have profound effects; these offer powerful targets for the understanding and manipulation of the system.

1.2 Powerful genetic approaches to perturb biological systems

The development of systems biology has been driven by a number of recent advances in our ability to perturb biological systems systematically. Three technological trends have emerged in this respect (26).

- Techniques for genetic manipulation have become more high-throughput, automated, and standardized by several orders of magnitude.
- The availability of complete genomic sequences has stimulated the development of systematic mutagenesis projects that complement more traditional efforts involving random mutagenesis.

- Technologies for disrupting genes in trans allow the application of genetic perturbations to a wide range of eukaryotic organisms.

1.3 Quantitative High-throughput Biological tools

The essence of the emerging systems biology approach is that, for any given species, the spatial distribution of biomolecules and their organization into pathways and processes is large but finite. The Human Genome Project has provided us with new technologies for systematically characterizing the cellular response: DNA sequencers, microarrays, and high-throughput proteomics. Typically, the development of these tools goes through three distinct stages:

(a) proof-of-principle

(b) development of a robust instrument

(c) the creation of an automated production line.

High-throughput DNA sequencing production lines may analyze genomic DNA and cDNAs as well as identify and type polymorphisms. Powerful sequencing techniques have emerged that allow up to 500,000 different sequences to be determined simultaneously for 16 to 20 residues (26). DNA arrays represent a second kind of powerful discovery tool. Two types of arrays are in common use: cDNA microarrays and oligonucleotide arrays. The former consist of double-stranded cDNA or PCR products spotted on a glass slide. The latter are synthesized or spotted on glass at densities that can exceed 50,000 spots per slide and are more specific than the cDNA array owing to their ability to distinguish single nucleotide differences. Proteomics, the characterization of the many

proteins within a cell type, involves analysis of different types of information corresponding to each protein species: protein identity, abundance, processing, chemical modifications, interactions, compartmentalization and turn over time. Perhaps, the major challenge of proteomics is to deal with the enormous dynamic range of protein abundances found in a single cell type – from 1 to 10^6 or greater. For organisms whose genome has been sequenced, mass spectrometry is an especially powerful tool for identifying and quantifying large numbers of proteins, identifying and typing single nucleotide polymorphisms (SNPs), and analyzing protein modifications.

Summarized below is a list of the evolution of protein chemistry methods and their adaptation from forward to reverse strategies (24). All these technologies can be integrated into a computational model.

- Discovery science: It comprises investigation of a biological system or process by enumerating the elements of a system irrespective of any hypothesis on how the system might function.
- 2DE: Two dimensional gel electrophoresis is the separation of proteins using two orthogonal parameters, isoelectric point (charge) and relative molecular mass, which are both usually determined on the basis of protein mobility in a polyacrylamide gel matrix.
- DNA microarrays: This comprises high-throughput differential screens of mRNA expression using complementary cDNA or oligoncleotide libraries that are printed in extremely high density on microchips. These microchips

are probed with a mixture of fluorescently tagged cDNAs, produced from two different cell populations and analyzed with a laser confocal scanner.

- Mass spectrometry: A mass spectrometer measures the mass-to-charge ratio of charged species under vacuum and is comprised of an ionization source and a mass analyzer. It can accurately provide mass measurement of charged analyte (ions). These analytes are usually peptides or less frequently protein ions.
- Protein identification: This is a method to determine the sequence identity of a protein; two common mass-spectrometry based approaches used are peptide mass mapping and searching uninterpretted MS/MS spectra; in both methods, observed data are matched to theoretically derived peptide and/or fragment ion masses calculated from sequence databases.
- Systems biology: It enables the study of a biological system by the systematic and quantitative analysis of all of the components that constitute the system.
- Yeast two-hybrid: It is a genetic-based method for identifying protein-protein interactions in vivo; a protein fused to the DNA-binding domain (bait) and a different protein fused to the activation domain of a transcriptional activator (the prey) are expressed in yeast cells; if an interaction between the bait and prey occurs, transcription of a reporter gene is induced and detected typically by a color reaction that indicates transactivation of the reporter gene.

- Phosphoproteomics: Proteo-mode enables observation and identification of hundreds of phosphoproteins for multiparameter analyses of complex phosphorylation networks- it is the integrated response of the network, expressed in terms of modifications in the form/amounts of many proteins that becomes the result of experimental analysis.

1.4 Computations for systems biology

1.4.1 Importance of global analysis and computer models

An enormous challenge for the future is how to integrate the different levels of information pertaining to genes, mRNAs, proteins and pathways. Given the recent accumulation of expression profiles, molecular interactions, and a variety of other global data in the biological databases, the immediate task is to develop powerful analyses and experimental strategies to integrate and analyze these data to make biological discoveries. Computer systems are required to store, catalogue, and condense the rapidly accumulating mass of data, and automated tools are needed that, by assimilating these data into a network model, can predict network behaviors and outcomes that maybe tested experimentally. It is encouraging that recent computer simulations of partial or whole genetic networks have demonstrated network behaviors, commonly called systems properties or emergent properties, that were not apparent from examination of a few isolated interactions alone (26).

Initially, signaling pathways were studied in a linear fashion, and it was shown that many important biological effects are obtained through linear information transfer.

However, it has become increasingly clear that signaling pathways interact with one another and the final biological response is shaped by interaction between pathways (27). These interactions result in networks that are quite complex and may have properties that are nonintuitive. A systematic analysis of interactions between different signaling pathways could be useful in understanding the properties of these networks. This increasing complexity of the signaling networks cannot be understood by experiments alone. The development of computational models and integration of these models with experiments will provide valuable insight into these complex systems-level behaviors (25). Computational models should represent the biological system as accurately as possible and be able to mimic the behavior of the system over a wide variety of conditions. For this to occur, the model should be based on and fully constrained by experimental data. Such data should include cellular concentrations of the components as well as the kinetic constants for interactions between components.

1.4.2 Types of computer models

A wide variety of cellular models have been proposed, each of differing complexity and abstraction. For example, chemical kinetic models attempt to represent a cellular process as a system of distinct chemical reactions. In this case, the network state is defined by the instantaneous quantity of each molecular species of interest in the cell, and molecular species may interact via one or more reactions. Often, each reaction is represented by a differential equation relating the quantity of reactants to the quantity of post-reaction products, according to a

reaction rate and other parameters. This system of differential equations is usually too complex to be solved explicitly, but given an initial network state, the quantity of each molecular species can be simulated to produce a state transition path or trajectory, i.e., the succession of states adopted by the network over time. Recently, it has been pointed out that transcription, translation and other cellular processes may not behave deterministically but instead are better modeled as random events (26). Models have been investigated that address this concern by abandoning differential equations in favor of stochastic relations to describe each chemical reaction.

In contrast to models involving systems of chemical reactions, another popular approach has been to model a genetic network as a simplified discrete circuit. Much like a neural network, this approach represents the network as a graph with nodes and arrows, where a node represents the quantity or level of a distinct molecular species and an arrow directed from one node to another represents the effect of the first node's level on that of the second. Also required is a function for each node, describing how all of the incoming effects should be combined to determine its level. Typically, nodes may assume one of two discrete levels, signifying whether the molecule is present or absent or whether a gene is turned on or of. In this way, the network state over all nodes evolves over a series of discrete time steps, where the state of the next step is computed from the current state (26). Proponents of discrete circuit models argue that they preserve the essential features of the underlying biology while greatly reducing network complexity and

simulation time. However, a major criticism has been that they require the model to update simultaneously for all nodes, whereas molecular interactions within the cell are not synchronous.

Formulation of a model involves important choices about which genes, gene products and other molecular species should be included in the network state. Ultimately, one wishes to understand the underlying interactions, molecular or otherwise, that are responsible for the global changes observed in a system. In order to most directly address this goal, it will be necessary to integrate the various levels of global measurements together and with a mathematical model of a biological system of interest.

1.5 Objective

In our study, we have used the movement of pigment granules in fish chromatophores as a tool to understand how perturbation of the G-protein mediated signaling at different points, by addition of diverse biologically active agents affects the cell response. Elicitor set approach is an experimental framework that has been employed in order to simplify the scrutiny of information flows through the signaling networks and elucidate the mechanism of action of an unknown agent which in our case is Phenylmethyl Sulphonyl Fluoride (PMSF). This method also aims to identify the existence of cross talk mechanisms between the different G-protein coupled receptor (GPCR) pathways. The fish chromatophores are sensitive to a variety of chemical/bioactive agents and provide the advantage of visualizing their response to different agents with a simple set-up.

2 LITERATURE REVIEW

2.1 The importance of organelle transport

Cells rely on efficient intracellular transport to position organelles and structures as large as chromosomes at the right place at the right time. These organized movements are known to support housekeeping as well as specialized cellular functions. Some examples of these movements are the transport of synaptic vesicles from the neuron cell body down the axon to the sites of release, the positioning of the golgi apparatus and lysosomes next to the nucleus, the pH-dependent dispersion of lysosomes, the trafficking between endocytic compartments, and the extension of endoplasmic reticulum and mitochondria towards the cell periphery. Organelle transport systems consist of cytoskeletal tracks and the associated motor proteins that actively move cargos along these tracks. There are three major components of cytoskeleton: intermediate filaments (8 – 10 nm), actin filaments (5 – 8 nm), and microtubules (25 nm). Actin filaments and microtubules are polar structures containing structurally different plus and minus ends, which allow directed movement of motor proteins associated with them. Motor proteins use the energy of ATP hydrolysis for directional movement. Motors are classified according to their interaction with the cytoskeleton, namely, actin filaments or microtubules, as well as according to their structural similarity. Myosins are plus-end directed motors that move along actin filaments and at least 14 different families of myosin have been described so far (18). There are two major classes of microtubule-based motors: dyneins and kinesins. Dyneins are

minus-end-directed microtubule motors, which were originally described in cilia and flagella and now are known to be in the cytoplasm of all eukaryotic cells. Kinesins comprise a superfamily of microtubule motors that share a highly conserved motor domain and are mostly plus-end directed. While kinesins move their cargo forward towards the periphery of the cell, dynein transports the granules towards the centrosome, located near the center of the cell.

2.2 Pigment cells: A model for the study of organelle transport

All eukaryotic cells rely on one or more intracellular transport systems. Pigment cells provide an excellent system for studies of organelle transport, because their main physiological task is to translocate pigment granules bidirectionally in response to defined chemical signals. Coordinated movement of granules towards the cell center or towards the periphery occurs in response to signal molecules (neurotransmitters or hormones) that bind to membrane receptors and trigger intracellular cascades. This system allows transport system in each direction to be experimentally induced and observed independently. The use of pigment cells for the study of intracellular transport started with the pioneering work of Porter and colleagues (18). Since then, pigment translocation in these specialized cells has been the object of extensive research. To date, pigment cells remain an extremely powerful model for the study of organelle transport and motor protein function and regulation.

Pigment cells (chromatophores) can be grossly divided into two categories: cells that undergo pigment redistribution or structural changes that result in

physiological color change (defined as rapid color changes based on the intracellular mobilization of pigment containing organelles), and cells that produce pigment that is transported to other cell types, which take it up, resulting in morphological color change (in which color changes result from changes in the number and synthetic activity of integumentary pigment cells) (18). It is common to use the term chromatophore to define all the cells that undergo fast physiological color change and to use the term melanocytes to refer to cells that play a role in long term morphological color change. While chromatophores including melanophores are found in many invertebrates and on the dermis of fishes, amphibians, and reptiles, melanocytes are found mostly in birds and mammals.

2.2.1 Types of chromatophores

Chromatophores are neuron-like cells that are classified according to the color of their pigment granules. Melanophores (brown to black), erythrophores (orange to red), and xanthophores (yellow to orange) have some characteristics in common: they are all derived from the neural crest, are located at the dermis, and are able to translocate pigment granules bidirectionally. At cellular level, these different chromatophores can regulate their color by having the pigment granules dispersed throughout the cytoplasm (dark) or by aggregating the pigment granules into a tight cluster in the center of the cell (pale) (9). Another class of chromatophores, called as iridophores (silvery) changes their color by an entirely different mechanism. They contain organelles consisting of stacks of purine crystals at very

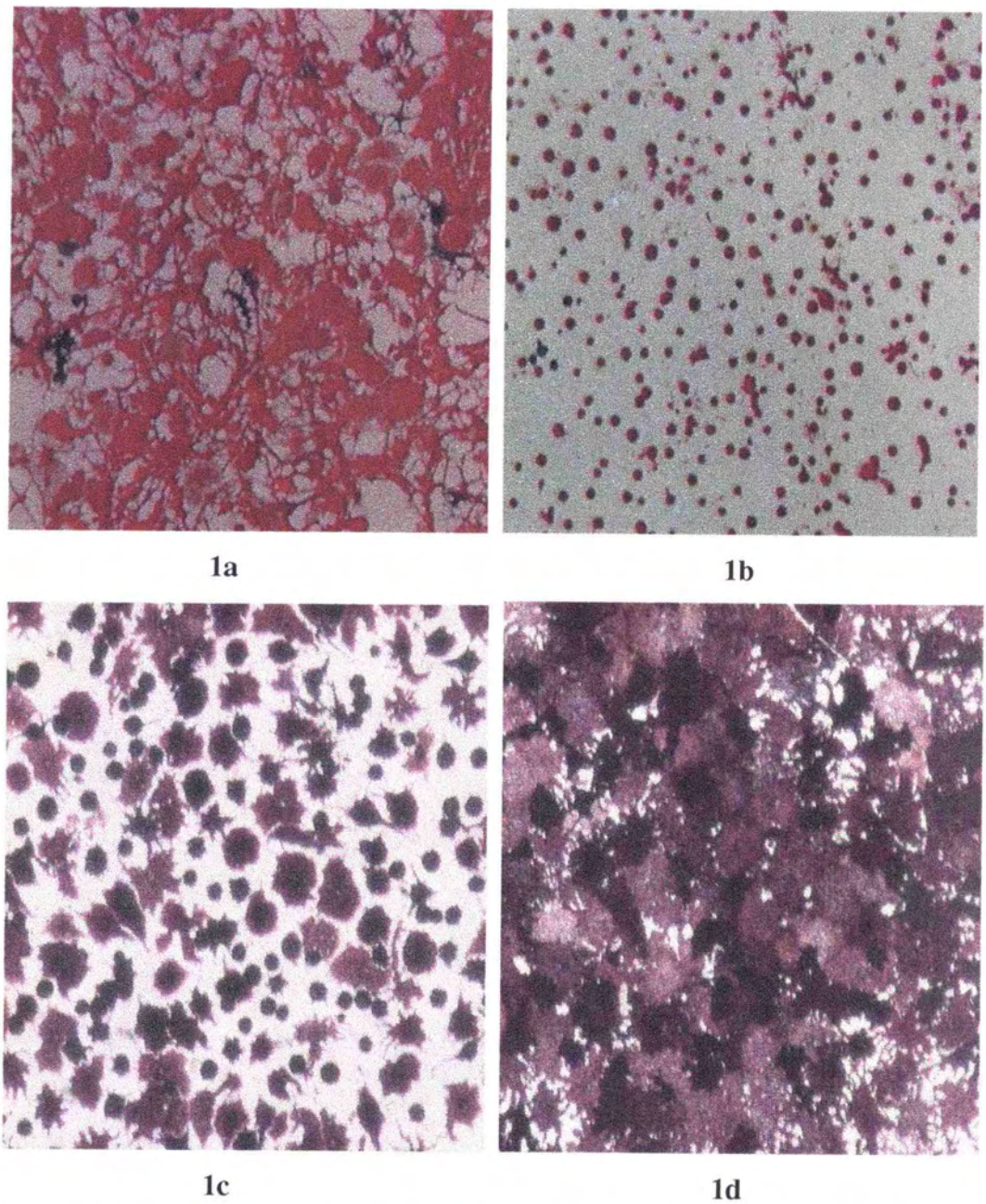
precise spacing designed to reflect light of a particular wavelength. Some types of iridophores can alter this spacing in response to neurotransmitters or other stimuli and thus change wavelengths of light reflected (22, 23).

2.2.2 Role of G-proteins in transport of chromatophores

Chromatophores contain various types of neurotransmitter and hormone receptors, depending on the species. The most important signal transduction mechanism linking agent receptor binding with pigment aggregation or dispersion is the classic G-protein-linked type. Receptors that cause aggregation are linked to G_i, whose activation results in a decrease in cAMP, while receptors that cause dispersion are linked to G_s, whose activation results in an increase in cAMP. The long range movements of pigment granules depend on polar microtubules and specific motor proteins bound to pigment granules (9). The ability of these motor proteins to bind microtubules resulting in the bidirectional transport of pigment granules is regulated by protein phosphorylation and dephosphorylation originating from the signal cascade initiated by G-protein-linked receptor binding. In addition to G_i and G_s, there is evidence that an increase/decrease in intracellular calcium levels mediated by G_q coupled receptors can also affect pigment granule movement. It has been recently shown that shorter-range granule movements depend on the interaction of myosin V and actin, and that this interaction is controlled by phosphorylation of myosin mediated by calcium/calmodulin dependent kinase II (9, 18).

Chromatophores have been an extremely useful reporter system to study G-protein-linked receptor/ligand interactions, as a tool for screening pharmacological agents and for dissecting the structure/function relationships of various ligands. The complexity of signal transduction agents affecting both long-range and short-range granule movements provides a powerful tool to detect the effects of agents that might perturb the pathway at a number of points, as well as agents that interfere more directly with microtubule transport and general cell metabolism. Inherent in the systems is the ability to monitor not only direct effects to perturb the normal state of the chromatophores, but also the ability to challenge the chromatophores with various agents and observe differences in the effected response (9).

Signal amplification can also be a source of increased sensitivity to certain agents. For example, a particular agent might completely inhibit aggregation, slow it down, or cause some sort of partial aggregation, depending on where it acted. Agents that interfere with glycolysis or the metabolism of ATP could affect granule movement by affecting the pool of ATP required for motor protein movement (9). Cross-talk among the signal transduction pathways can also increase the repertoire of agents to which the cells are sensitive. There is increasing evidence that macromolecular signaling complexes are formed within cells that can give rise to chemical compartmentalization, an important consideration when observing large dendritic chromatophores.



1a **1b**

1c **1d**

Figure 1: Pigment granule distribution in red *Betta splendens*

The figure 1 above shows chromatophores in both dispersed and aggregated state. The field of view is primarily erythrophores with a few melanophores. The figures 1a, 1b were taken before and after challenging the cells with 100 nM clonidine, a

G_i protein activator. The figures 1c, 1d demonstrate the before and after state of the chromatophores in response to 10 μM forskolin, an adenyl cyclase activator. The cells were exposed to the agents for 15 minutes. 10X magnification was used to capture the images and while the clonidine experiments were conducted using fiber optic light source, forskolin experiments were conducted using the light source on the microscope .

2.2.3 Co-operative effect of actin and microtubule motors

To explain the need for two separate cytoskeletal systems for transport of pigments, there are two hypotheses, which are not mutually exclusive (18). First, a plus-end microtubule motor is responsible for fast outward movement, and a myosin is required to promote even distribution of pigments throughout the cell. In this model, disruption of actin leads to hyper dispersion due to action of a strong outward microtubule motor. This model is well illustrated by the results obtained in fish melanophores. Second, microtubules would be responsible for long-range movements while actin filaments would trap the pigment organelles at the periphery of the cell. Therefore, in the absence of actin based movement, pigment granules can no longer accumulate in the periphery. Both these ideas may be correct. Myosin, being a highly processive motor, could be responsible for local movements in random directions, promoting even pigment distribution, as well as for trapping organelles at the cell periphery, as it stays bound to actin most of the time. The differences in the speed of movement of the pigment granules observed among fish, frog and mammalian systems can be attributed to the relative

contributions and efficiencies of the microtubule motors rather than to distinct roles of myosin V. Cooperation between actin and microtubule motors has been previously indicated by the observations that some organelles move along both these cytoskeletal tracks. These observations led Langford to propose the 'dual filament model of transport', in which microtubules provide the tracks for long distance movement and actin filaments provide the track for local movement (14).

2.3 Bidirectional movement of chromatophores by G_s/G_i activation

2.3.1 Dispersion

A model for pigment aggregation and dispersion is shown in figure 2.

This model predicts that changes in intracellular levels of cAMP regulates bidirectional movements in melanophores. The process of pigment granule dispersion can be stimulated by β-adrenergic agonists, melanocyte-stimulating hormone (MSH), and adrenocorticotropin hormone (ACTH) which is structurally related to MSH. Subsequent to hormone receptor binding, adenyl cyclase (AC) activity is stimulated with a concomitant elevation in intracellular cAMP concentrations. Binding of cAMP to the regulatory subunits of cAMP dependent protein kinase A (PKA) results in the release and activation of its catalytic subunits. Using ATP as a substrate, a high level of protein phosphorylation of protein X, potentially the 57 kd polypeptide or other polypeptides is initiated by the activated protein kinase (21). As indicated by multiple arrows in figure 2, following this phosphorylation, the process of dispersion may be activated as an end result of a poorly understood chain of events.

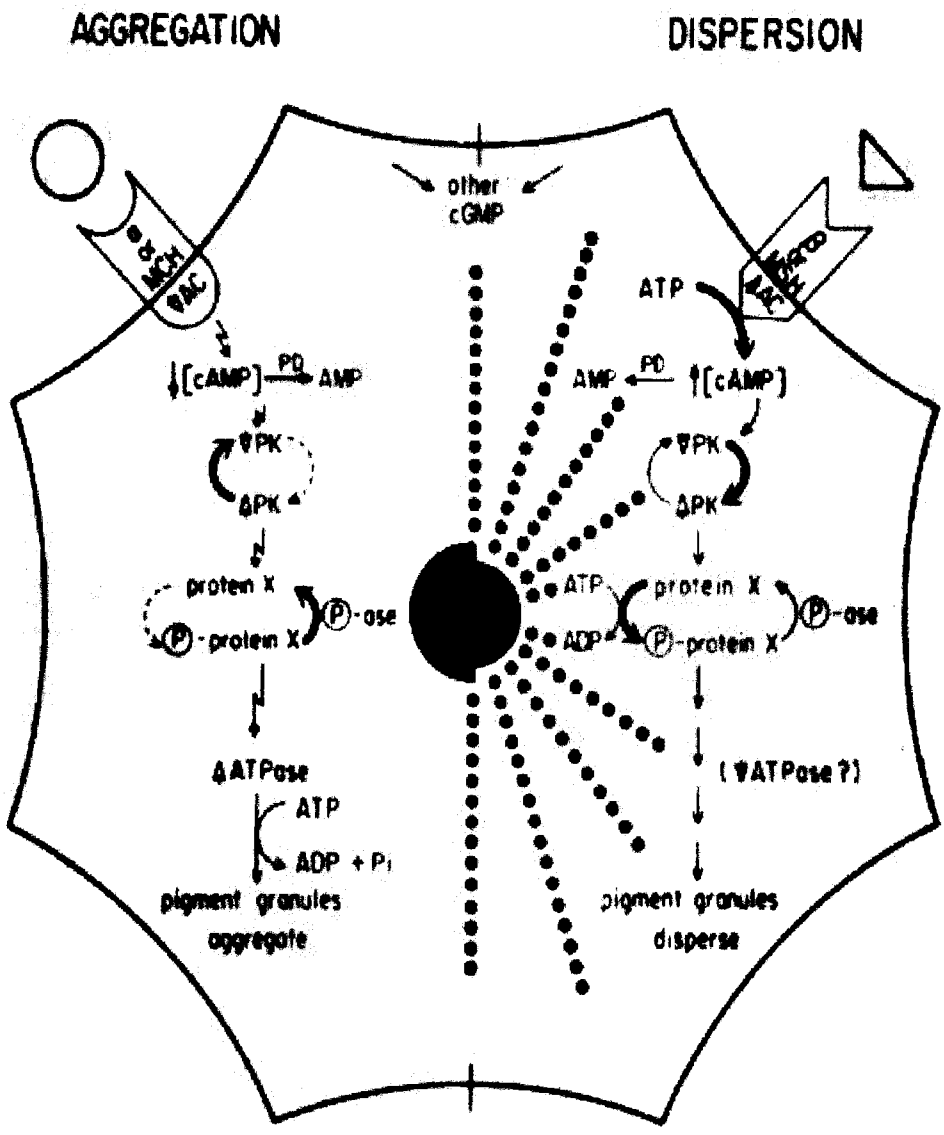


Figure 2: Cellular signaling mediated through the cAMP pathway. Activation of the G_s and G_i pathway in response to Melanocyte Stimulating Hormone (MSH) and Melanin Concentrating Hormone (MCH) respectively in concentrations of

nanomolar range. Stimulation of the G_s cascade results in dispersion while G_i activation induces aggregation.

2.3.2 Aggregation

Pigment granule aggregation is initiated after addition of α-adrenergic agonists or melanin-concentrating hormone (MCH). These hormones decrease intracellular cAMP levels by inhibiting adenyl cyclase and stimulating phosphodiesterase activity respectively. Lowered cAMP levels result in the reduced activity of cAMP-dependent protein kinase by promoting the formation of the inactive holoenzyme (Figure 2, broken arrows indicate indirect reaction steps) (21). Subsequently the concentration of phosphorylated protein X, required for cells to be in a dispersed state, is reduced by cellular phosphatase.

2.3.3 Pigment transport regulation by phosphorylation/dephosphorylation

The induction of pigment granule dispersion and aggregation in melanophores is regulated by protein phosphorylation and dephosphorylation. A 57 kd polypeptide was the only phosphoprotein detected by one dimensional gel analysis whose dephosphorylation/phosphorylation correlated with pigment aggregation and dispersion (21). Phosphorylation of this protein was stimulated several fold during pigment dispersion and the same protein was dephosphorylated during pigment aggregation in melanophores from the African cichlid, *Tilapia mossambica*. PKI inhibited both pigment dispersion and phosphorylation of the 57 kd polypeptide, while vanadate inhibited both pigment aggregation and dephosphorylation of this

protein. Accordingly, the phosphorylation state of the protein may determine the direction of pigment transport.

2.3.4 PKA and A kinase anchoring proteins (AKAPS)

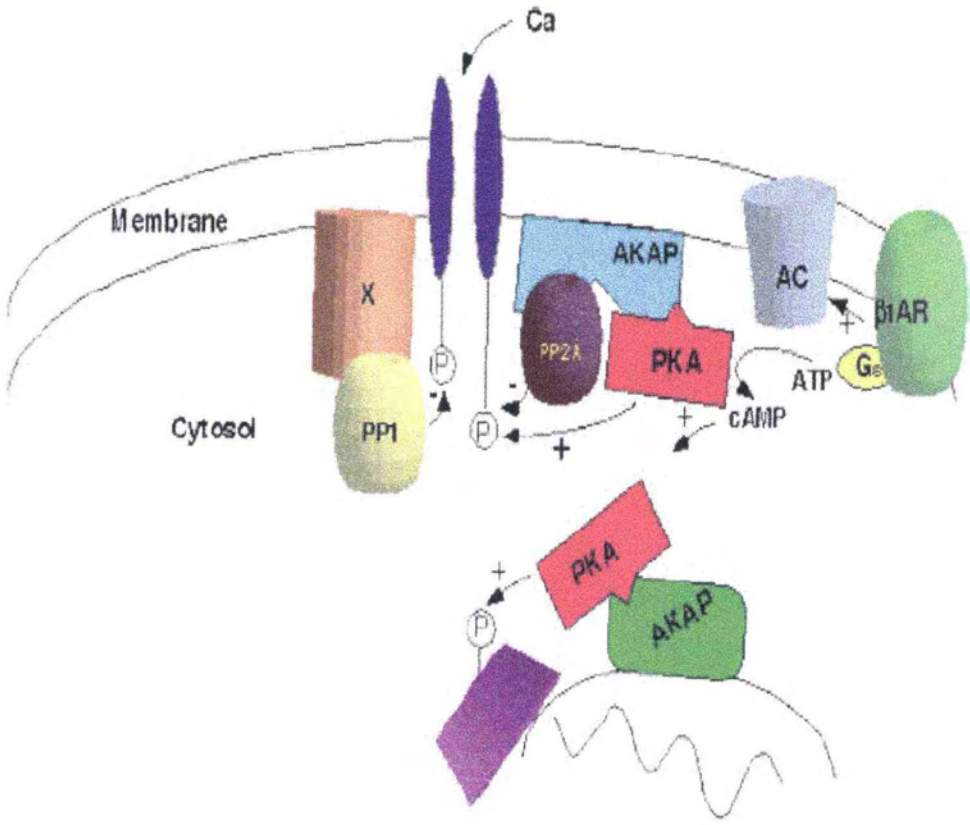


Figure 3: Schematic representation on the role of PKA anchoring to their AKAPs in determining substrate specificity. Shown PKA (*red*) and two AKAPS (*light blue and green*). Upon activation of the β-adrenergic receptor, which couples to adenylyl cyclase via the G_s protein, cAMP is generated. cAMP causes the release of the catalytic subunit of PKA from the AKAP at two distinct subcellular sites. The distinct localization of each activated PKA by anchoring two AKAPS at

different subcellular sites renders them proximal to different substrates; the first AKAP is located near the L-type calcium channel (*blue*) and thus regulates its function, whereas the other is localized on the mitochondrial membrane adjacent to a different substrate (*purple*). In addition, AKAP can anchor a selective phosphatase, PP2A (*dark purple*) to the same site, leading to synchronized phosphorylation and dephosphorylation of the channel.

A family of anchoring proteins, termed AKAPs for A kinase anchoring proteins, binds inactive PKA via its regulatory subunit and localizes it to distinct subcellular sites. There are multiple AKAPs in each cell type, each localized to different subcellular sites. Upon elevation of cAMP, the catalytic subunit is released and phosphorylates nearby substrates. Previous studies have demonstrated that disruption of PKA anchoring to AKAP via a peptide derived from one of the AKAPs inhibits its function in the cell (10). This peptide, Ht31, is not selective for individual AKAPs. However, it was effective in demonstrating the role of anchoring of PKA to AKAP in PKA mediated signaling.

2.4 G_q mediated signaling and PKC anchoring

It is interesting to note that activation of α1-adrenergic receptor results in dispersion of the frog chromatophores (1), but aggregation of the fish chromatophores (9). The active α1-receptor in turn activates phospholipase C (PLC), with which it is coupled via the G_q protein. PLC causes the hydrolysis of phosphotidylinositol 4-5 bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 causes release of Ca^{2+} from the endoplasmic reticulum.

The DAG produced from PIP2 is transient and it is frequently followed by a more sustained increase following the hydrolysis of phosphatydylcholine (PC) by phospholipase D (PLD) (2). High levels of intracellular calcium ions in the cytosol cause PKC to bind to the cytosolic leaf of the plasma membrane, where it is activated by DAG. This activation results in an array of cell responses through the activation of cell-specific transcription factors (e.g., c-Fos, c-Jun. NF-κB) (15). In fish chromatophores, PKC activation results in aggregation of the pigment granules.

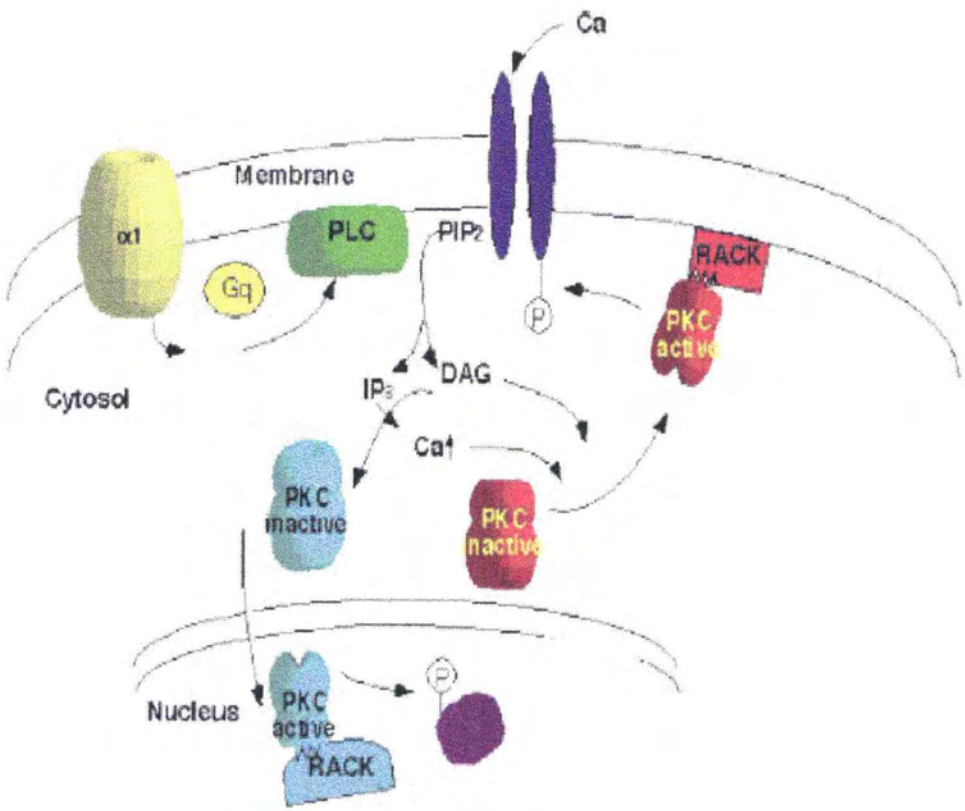


Figure 4: Schematic representation on the role of PKC anchoring to their RACKs in determining substrate specificity. Shown are two PKC isozymes (*red and light*

blue) and their corresponding RACKs. On activation of α1-adrenergic receptor that couples to phospholipase C (PLC) via the G_q protein, both diacylglycerol (DAG) and inositol triphosphate (IP_3) are generated. IP_3 increases the intracellular calcium ions in the cytosol and, together with DAG, causes translocation of the PKC isozymes to distinct subcellular sites. One PKC isozyme (*red*) translocates from the cell body to the plasma membrane, whereas the other (*light blue*) translocates into the nucleus. The distinct localization of each activated isozyme to different subcellular sites brings them close to different set of substrates; whereas the red isozyme is located nearby the L-type calcium channel (*dark blue*) and thus regulates its function, the activated blue isozyme is localized near nuclear proteins and thus may regulate gene expression by phosphorylation of, for example, a transcription factor (*purple*).

Several studies have demonstrated that each PKC isozyme is localized to distinct subcellular sites both before and after activation (10). At the time that these observations were made, activated PKC isozymes were all assumed to be associated with plasma membranes. The rationale for that notion was that DAG, which is necessary for activation of most PKCs, is derived from membrane phospholipids. Activation of PKC in cells is accompanied by translocation of the enzyme from the cell soluble fraction to the cell particulate fraction, which is enriched with plasma membranes. It was, therefore, natural to assume that activation of PKCs induces translocation of cytosolic soluble enzymes to the

plasma membrane. It also followed that localization of activated PKC isozymes would be determined solely by lipid-protein interactions.

However, because of the observed distinct subcellular location of PKC isozymes, it was suggested that localization of each activated PKC isozyme must also require protein-protein interactions between each activated isozyme and a specific, isozyme-selective anchoring protein, which were collectively termed receptors for activated C-kinase (RACKs). The existence of a different RACK for each PKC isozyme was hypothesized. The anchored RACK binds its specific activated PKC isozyme in a locale containing a defined subset of protein substrates and away from others, which is the determining factor for substrate specificity of that isozyme (see Figure 4 for a schematic presentation). In addition, based on immunofluorescence studies, it was suggested that inactive PKCs must also be anchored via protein protein interactions.

2.5 Possible roles of the three motors involved in pigment migration

Experimental evidence suggests that phosphorylation mediated by PKA induces dispersion in vivo by inactivating dynein - and activating kinesin II – microtubule interactions so that plus-end directed microtubule transport is favored (8,22). Protein phosphatase 2 A (PP2A) has the opposite effect on the motors' microtubule binding properties as PKA and dephosphorylation by PP2A was found to induce aggregation in vivo by inactivating kinesin II – and activating dynein-microtubule interactions so that minus end directed transport was favored. Similar studies were conducted to investigate dynactin-microtubule binding which

was found to be inhibited by PKC and enhanced by PP1 (8). PKC activation in fish chromatophores causes aggregation while its inhibition results in dispersion. During pigment granules transport, whether these 3 motors themselves are phosphorylated is still not known, although evidence suggests that neither cytosolic kinesin II nor cytoplasmic dynein are differentially phosphorylated during pigment dispersion or aggregation (18). Other putative targets for regulation are the membrane receptors for motors. Identification of the target proteins for phosphorylation is an important step in filling the gaps in our understanding of regulation of pigment transport. Efforts have to be directed towards investigating how the motors are regulated in such a way that proper distribution of pigments is precisely achieved in spatial and temporal dimensions in these pigment cells.

2.6 Cross talk at the effector level

The downstream activation of kinases [e.g. PKA and PKC] that can phosphorylate both receptors and effectors introduces a number of potential sites of both stimulatory and inhibitory cross-talk. In the following sections, we discuss the multiple mechanisms of cross-talk between receptors coupled to different G-proteins and address the limitations within which they may occur (28).

2.6.1 Between G_s and G_q coupled receptors

The signaling pathways activated by G_s and G_q proteins lend themselves to cross-talk at numerous levels, as summarized in figure 5.

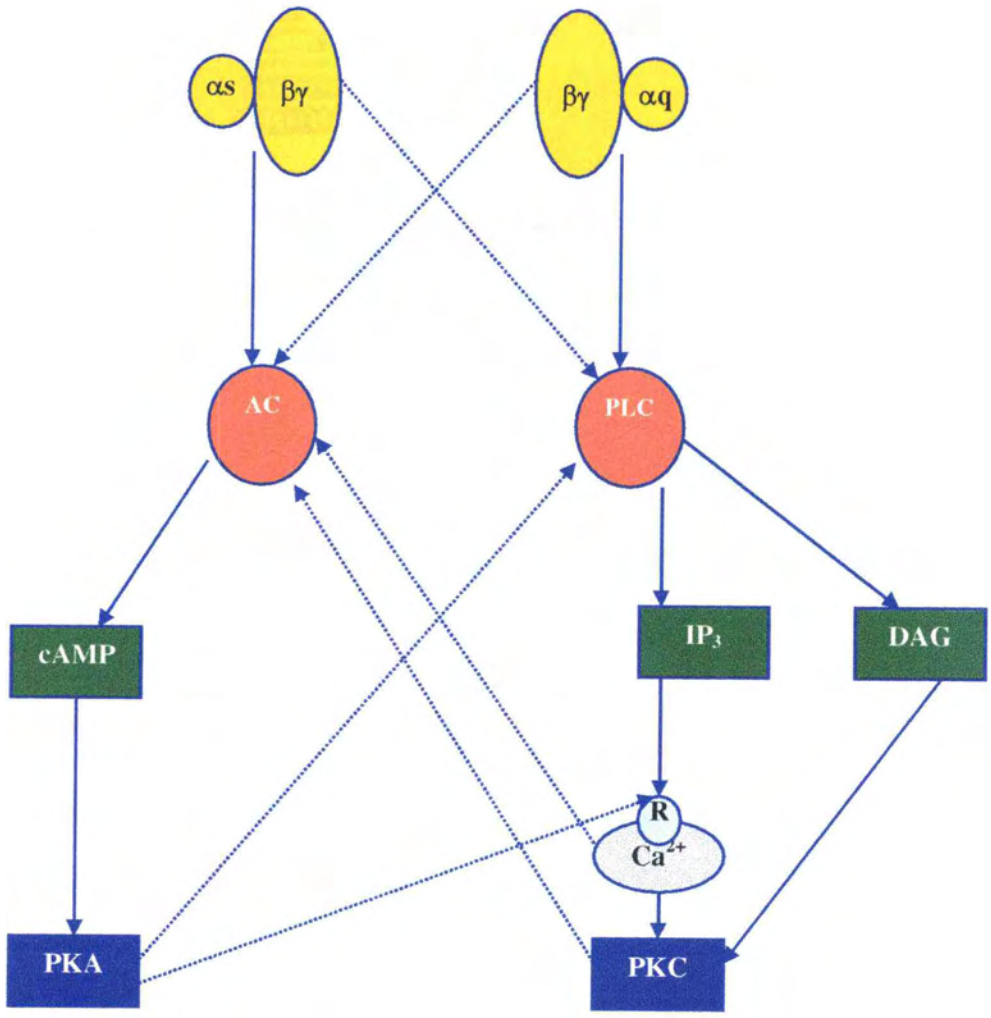


Figure 5: Cross-talk between G_s and G_q pathways

- βγ subunits from G_s may stimulate PLC, potentiating a G_q-mediated response.
- βγ subunits from G_q may stimulate or inhibit particular adenyl cyclase (AC) isoforms, thus potentiating or hindering a G_s-mediated response.
- PKA may phosphorylate and deactivate PLC.

- PKA may phosphorylate and modulate the activity of IP_3 receptors.
- PKC may phosphorylate and activate isoforms of AC.
- Calcium ions may either activate or inactivate isoforms of AC.

2.6.2 Between G_i and G_q coupled receptors

The cross-talk occurring between the G_i and G_q proteins is depicted in figure 6.

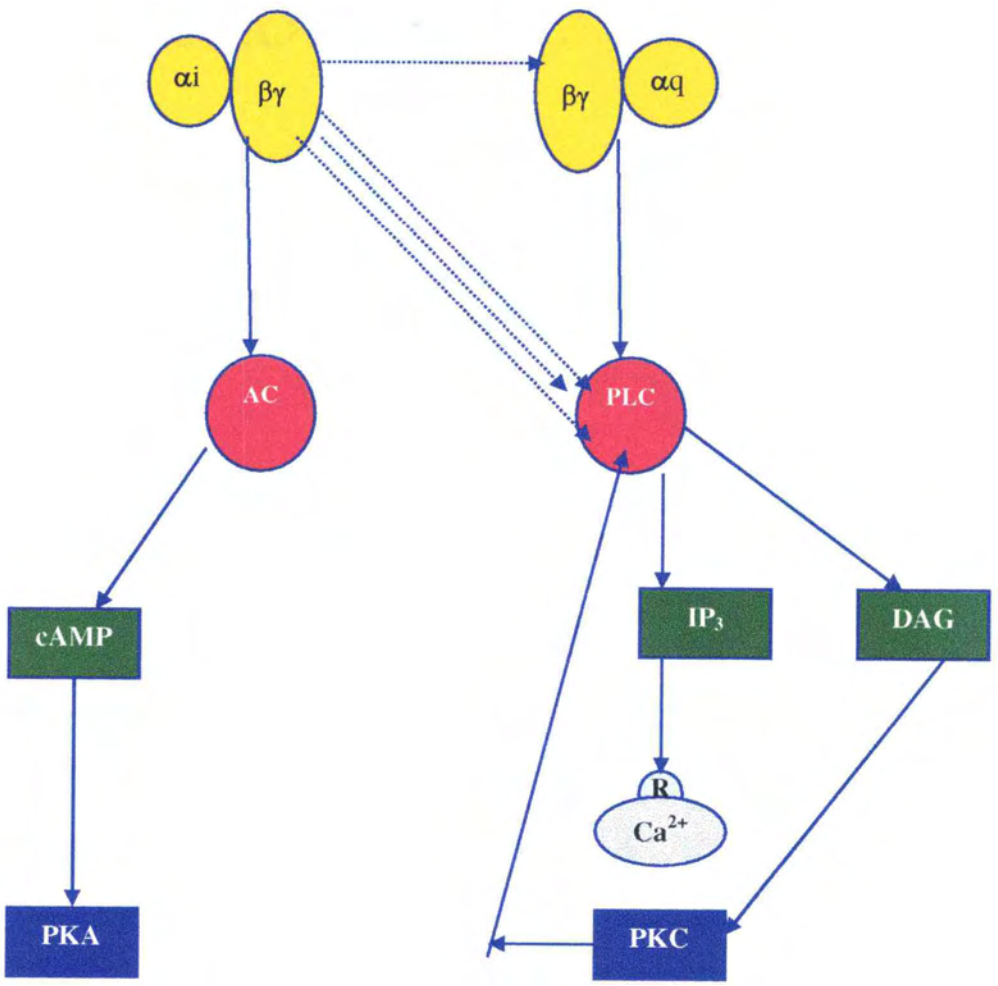


Figure 6: Cross-talk between G_i and G_q pathways

- βγ subunits may also be exchanged between G_i and G_q.
- βγ subunits released following G_i activation also stimulate PLC and potentiate responses mediated by G_q. βγ subunits may increase G_q activity by reducing the ability of PLC to accelerate GTP hydrolysis.
- Phosphorylation and inactivation of PLC by PKC may be inhibited by βγ subunits of G_i.

2.7 Why *Betta splendens*

Many species of fish contain motile chromatophores including the popular medaka, angelfish, goldfish, zebrafish and various African cichlids such as tilapia and jewel cichlid. Fish chromatophores are terminally differentiated and do not replicate in tissue culture. The viability of chromatophores in primary tissue culture is up to 3 months. Their energy requirements in the ground state appear to be small and hence, it is possible to maintain good health of the cells with minimum media change. The main disadvantage in using primary tissue culture is the need to tackle fish-to-fish variation in the various subpopulations of chromatophore types. We have also documented individual variations in responses to various agents. However, I have made every effort to use cultures that have been tested for similar responses.

A primary tissue culture protocol has been developed and the chromatophores from a large number of species have been isolated in order to compare their culture characteristics (9). *Betta splendens* chromatophores are relatively small (50 μm) and can tolerate close contact. For example 1000 *Betta splendens* chromatophores

can be cultured in an area of less than 1 mm^2. In contrast, only 10 tilapia chromatophores can be cultured in the same area, not only because of their larger size but also because they fail to survive at high densities. Cultured chromatophores from *Betta splendens* can thrive for at least a month. Bettas come in a variety of colors and are readily available from commercial sources. Their fins are densely packed with chromatophores, giving them their vivid colors. In tissue culture, the chromatophores tend to recreate their close proximity in the fin, providing high cell densities important for optimal cell response and image processing.

3 EXPERIMENTAL PROCEDURES

3.1 Salient features of elicitor space approach

Elicitor space experiments involve using a combination of agents and effectors that act at different points along the signaling cascade.

What is an elicitor?

Elicitor = effector (e.g. clonidine) + application method (100 nm clonidine added half an hour after incubation with unknown agent).

Why elicitor sets?

- Enable identification of the key nodes in the signal transduction pathway
- Segregation of the pathway into different modules that are individually constrained by experimental data
- Perturbation of the signaling cascade by adding different effectors will help investigate the cross-talk mechanisms.

3.2 Materials and Methods

Clonidine, Cirazoline, H-89, Forskolin, MSH, and PMSF were purchased from Sigma. Cell culture reagents were purchased from Gibco Invitrogen and the 24-well plates were obtained from Corning. The plate reader for optical density measurements was acquired from Bio-Rad Laboratories (Hercules, CA). The water bath used for warming solutions was a 180 series one from Precision and the centrifuge model was 5810 R from Eppendorf. Fish chromatophores from red *Betta splendens* were cultured in L-15 medium containing 20 mM Hepes, 100 units/ml penicillin, 100 mg/ml streptomycin, 2.5 mg/ml fungizone and 5 % Fetal

Bovine Serum (FBS). Please refer Appendix A for tissue culture protocol. The cultured cells were plated in polystyrene Costar 24 well plates and old media was replaced with fresh media one day after the culture. Experiments were conducted on the following day, once the cells had spread out after the media change. 10 mM stock solutions were prepared for forskolin, H-89, and clonodine, the first two in DMSO and the third in water respectively. 20 mM Cirazoline, 1 mM MSH (Melanocyte stimulating hormone) and 200 mM PMSF (Phenylmethanesulfonyl fluoride) stock solutions were prepared, the first two in water and the third in 100% ethanol respectively. The cell response was quantified by obtaining optical density measurements and data analysis was done by plotting normalized percentage change in optical density versus time.

3.3 Dilution Curve Experiments

Dose response experiments were performed for each of the agents at concentrations as listed below.

Clonodine: 500 nM, 100 nM, 10 nM

Cirazoline: 100 μM, 10 μM, 1 μM

H-89 : 100 μM, 10 μM, 1 μM

Forskolin: 100 μM, 10 μM, 1 μM

MSH: 10 nM, 1 nM, 0.1 nM

Controls:

L-15

DMSO control for Forskolin

All experiments were done in triplicates. 2X concentrations of the agents/effectors were prepared by appropriately diluting the respective stock solutions. Please refer to Appendix B for stock solution preparations. 1 ml of the 2X concentration was added to the cell well containing 1 ml of media so as to achieve the final concentration. The direct effect of each of these agents on the cells can be visualized under the microscope in terms of the cells either dispersing or aggregating. There are two methods that can be adopted to quantify the cell response to varying concentrations of the agents and the methods have been explained in detail in the appendix C. In our experiments, a plate reader has been used to measure the optical density of the cells before and after addition of the agents (clonidine, cirazoline, forskolin, MSH) at regular time intervals for a total time length of 51-52 minutes. H-89 dilution curves alone were obtained after 18 minutes of monitoring with a plate reader. Thus, the cell response at various time points can be obtained, with the ground state condition of the cells being measured at zero time before agent addition. Normalized optical density values versus time are plotted for a range of concentrations of each of the agents. The dilution curves thus obtained will explain the nature of response that each agent/effector elicits. For example, the aggregation due to clonidine addition yields a curve with a decreasing trend, whereas, in the case of dispersion caused by forskolin, the curve will show an increasing trend. After reaching a maximum or minimum, the curve tends to level off, indicating that a steady state has been reached.

The dose response experiments enable us to choose appropriate effector concentrations for formulating the secondary elicitor panel. The results of the dilution curve experiments showed that 10 μM forskolin, 1 nM MSH, 100 nM clonidine, 100 μM cirazoline and 100 μM H-89 yielded a strong direct response and hence, these concentrations were chosen while formulating the elicitor panel. 1 μM cirazoline and 1 μM H-89 which hardly caused any direct response in the dose response experiments were also included as secondary elicitors to observe if they would elicit an interesting response when applied along with the unknown agent.

3.4 Elicitor Set Experiments

Primary Elicitor: 10 μM Forskolin

Unknown agent: 1 mM PMSF

Secondary Elicitor Panel: 1 μM and 100 μM cirazoline, 1 μM and 100 μM H89, and 100 nm Clonodine.

Choice of PMSF as "unknown agent"

- PMSF is a fairly well characterized chemical agent. It is a serine protease inhibitor
- Interesting agent that plays contradicting roles in organo-phosphate induced delayed neuropathy depending upon the sequence of dosing

  Organophosphate-induced delayed polyneuropathy (OPIDP) is a rare toxicity caused by certain organophosphorous esters (OPs) like diisopropyl phosphorofluoridate (DFP), in several species and is characterized by axonal degeneration of some long axons in the central and peripheral

nervous system. The first step in the initiation of OPIDP is proposed to be the phosphorylation of an enzyme found in the nervous system called neurotoxic esterase (neuropathy target esterase, NTE) (16). Certain sulphonates, like PMSF, when given prior to neuropathic doses of organophosphates, protect hens from OPIDP and the protection was related to the inhibition of the putative target of OPIDP, which is the NTE (4). However, when administered in hens after a non-neuropathic dosage of OP, this non-neuropathic inhibitor, PMSF, can intensify or potentiate signs of OPIDP. The promotion of OPIDP was more severe in birds when PMSF was given after prior exposure to higher doses of OPs. Studies are being done to determine how exactly PMSF potentiates OPIDP. As both DFP (2 mg/kg) and PMSF (90 mg/kg) cause greater than 90% inhibition of NTE activities, the differential ability of PMSF to exacerbate delayed neurotoxicity suggests that it is interacting with a target other than NTE (4). The identification and characterization of this molecule may provide critical insight into the cellular responses elicited by neuropathic organophosphates and aid in the elucidation of pathological mechanisms involved in OPIDP.

- PMSF is known to have an effect on pigment granules transport and acts independent of the calcium signaling pathway. Previous experiments have demonstrated that PMSF causes a freezing effect after long term incubation, but the mechanism of action is not known.

- Previous experiments have shown that PMSF causes a shift in elicitor space and improves cluster separation between Bacillus cereus strains 1, 5 and 6.
- PMSF can be used as a model compound along with elicitors, for mechanistic classification, to explain the differences in cell response.

The cells were first exposed to a final concentration of 10 μM forskolin for 24 minutes followed by a final concentration of 1 mM PMSF for 2.77 hours. This long time period of exposure is because PMSF acts by diffusing across the cell membrane into the cells and this is a slowly occurring process. At the end of 2.77 hours, the cells were treated with the secondary elicitor panel for 42 minutes. All experiments were performed in triplicates. Since stock solution of forskolin was prepared in DMSO and that of PMSF was prepared in ethanol, there were two controls:

DMSO + EtOH + Elicitors

Forskolin + EtOH + Elicitors

Optical density measurements were taken before and after addition of each of the agents and effectors at regular time intervals using a BioRad plate reader. Preliminary experiments were done without PMSF, wherein the cells were exposed to the primary elicitor for 18 minutes, followed by exposure to the secondary elicitors for another 19 minutes. In addition to 10 μM forskolin, 1 nM MSH was also used as a primary elicitor and experiments were done in duplicates. DMSO and L-15 controls were included. Forskolin and MSH act at different

points along the G-protein signal transduction cascade although both result in dispersion response in the fish cells. These experiments were performed to demonstrate that the elicitor set method accomplishes segmentation of the signaling pathway. The elicitor sets experimental protocol has been explained in detail in appendix D.

4 RESULTS AND DISCUSSIONS

The figures in this chapter have been obtained by plotting the average of the normalized percentage change in optical density versus time. For the dose response experiments and PMSF-elicitor experiments, an average over triplicates was obtained while for elicitor experiments without PMSF, an average over duplicates was obtained.

4.1 Dose response experiments

The results of the dilution curve experiments for the different elicitors, both primary and secondary are depicted in figures 7, 8 and 9 along with their controls. Clonidine binds to the α2-adrenergic receptor, resulting in the activation of the G_i protein, thus leading to aggregation of the chromatophores and hence, a subsequent decrease in the optical density values. Cirazoline targets the α1-adrenergic receptor, causing the activation of the G_q protein. Subsequent elevations in intracellular calcium ion levels also result in aggregation of the fish chromatophores, which has been interpreted in terms of the decreasing values of optical density. The L-15 controls have hardly caused any change to the physical state of the cells as can be seen from the graph above. At the end of 51 minutes, the percentage change in optical density values in cells treated with plain L-15 was found to be 98%. Both clonidine and cirazoline exhibited a dose response pattern, as the decrease in optical density increases with an increase in the concentration, as shown in figure 7. While 500 nM clonidine results in 48% reduction in optical density after a duration of 51 minutes, 100 nM and 10 nM clonidine result in 61%

and 84% reduction respectively, within the same time limit. In the case of cirazoline, it was found that, at concentrations of 100 μM, 10 μM, and 1 μM, the corresponding decrease in optical density values 51 minutes after addition, amounted to 61%, 77% and 84% respectively.

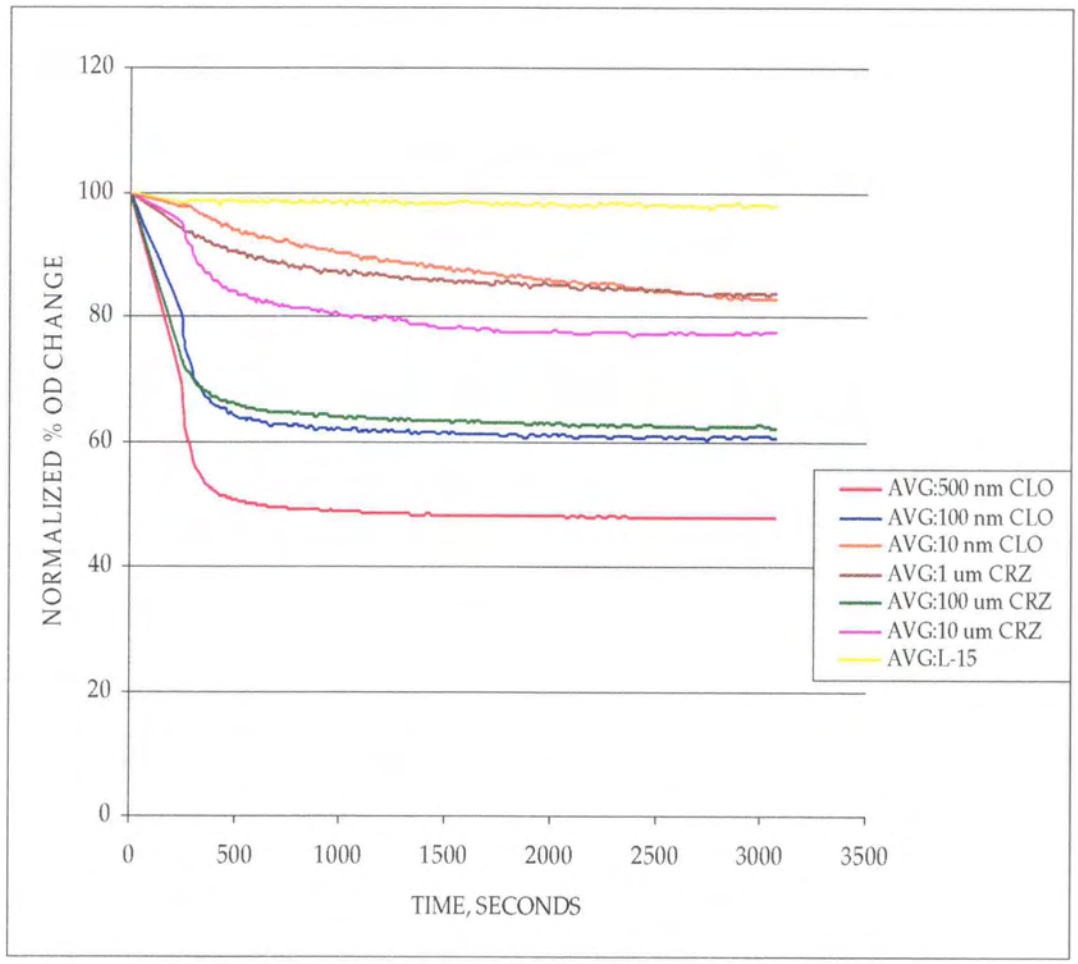


Figure 7: Dilution curves for Clonidine, Cirazoline and L-15 control

H-89 has been widely used as a specific inhibitor of protein kinase A. Inhibition of PKA hinders the phosphorylation and thus promotes dephosphorylation of the downstream target proteins, thus resulting in rapid aggregation of the

chromatophores. Literature reports suggest that H-89 is effective only at higher concentrations, between 100 µM and 200 µM, which are in agreement with our experimental data.

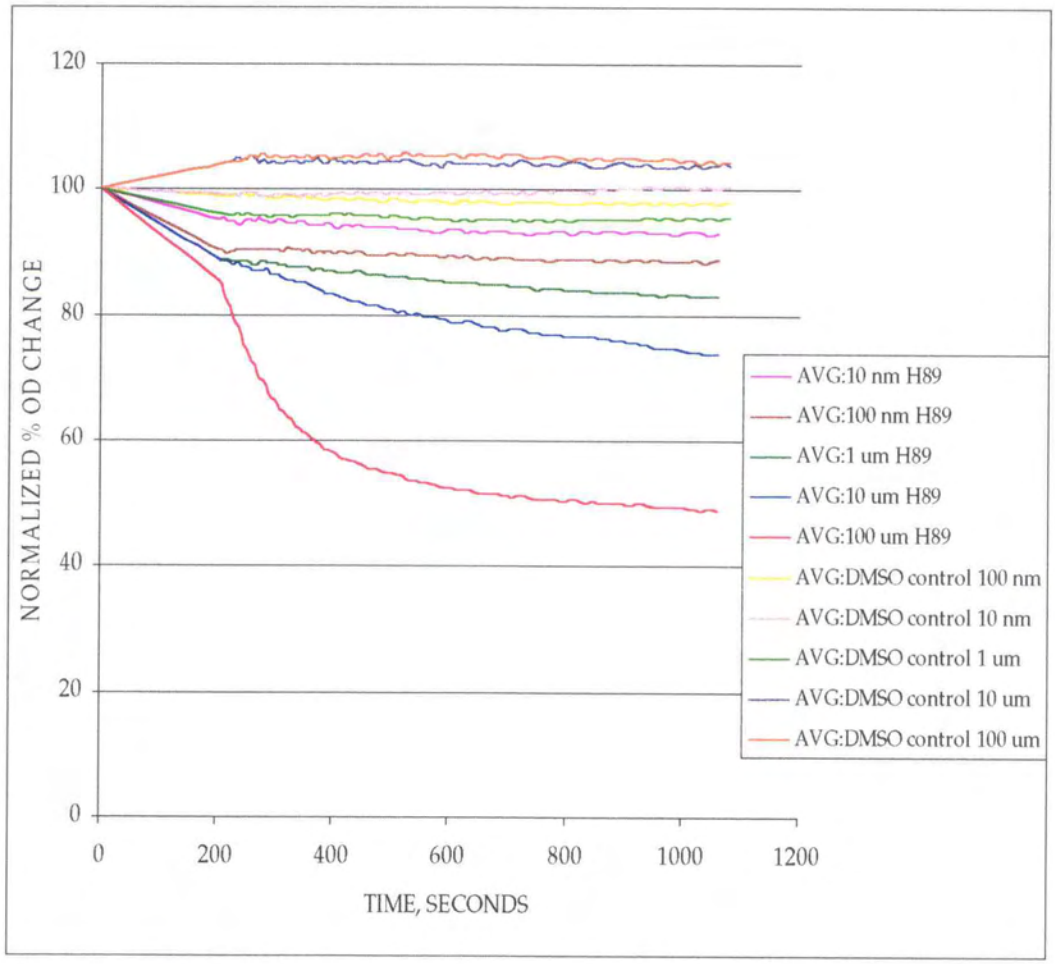


Figure 8: Dose response curves for H-89 and DMSO controls

It can be inferred from figure 8 that, at lower concentrations of 10 nM, 100 nM, 1 µM, and 10 µM, H-89 is found to cause little or slight aggregation, as can be seen from the percentage change in the optical density values, which were 93%, 89%, 83% and 74% respectively. The effect of H-89 is highly pronounced at 100 µM,

wherein, the optical density decreased to 49% of its value at ground state in a time length of 18 minutes. In cells treated with varying concentrations of DMSO, the optical density values varied between 95% and 104% respectively, which confirms that the decrease in percentage of optical density obtained at 100 µM concentration of H-89 is solely due to the effect of H-89 itself, and not because of the DMSO present in the stock solution.

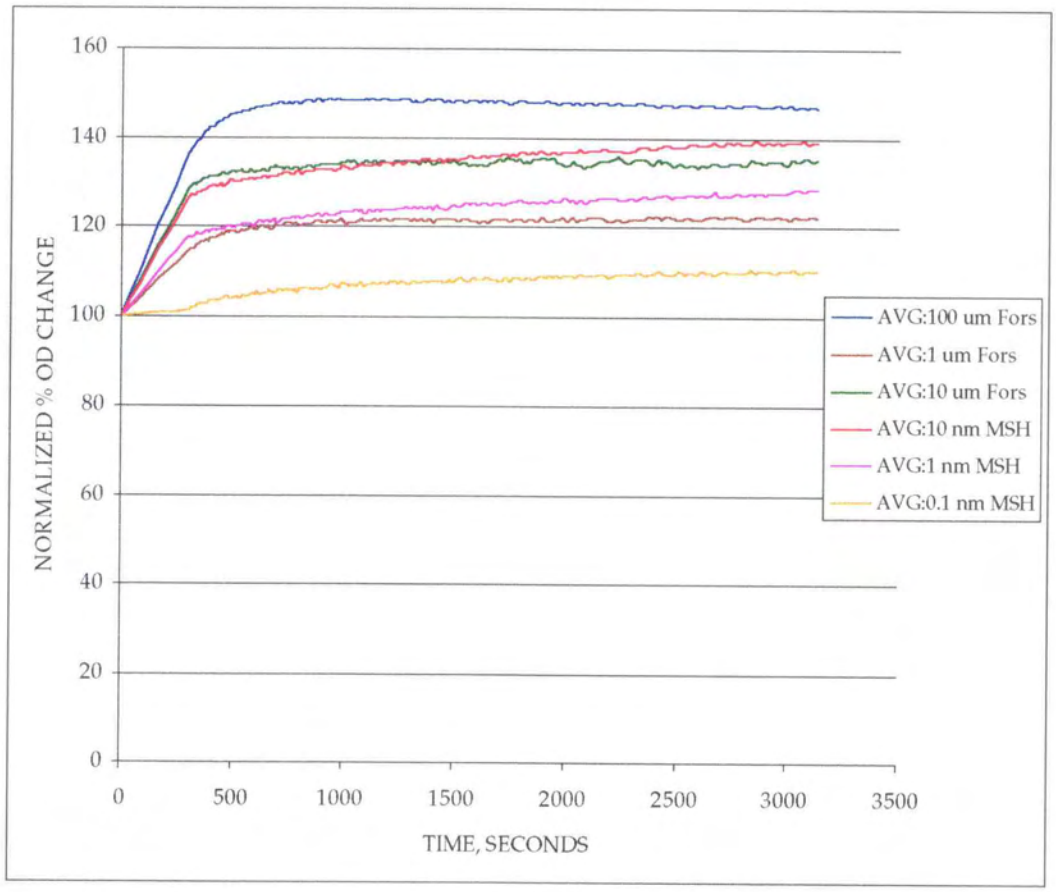


Figure 9: Dilution curves for Forskolin and MSH

Forskolin constitutively activates the enzyme, adenyl cylase resulting in an elevation in cAMP levels and subsequent stimulation of PKA. Activation of PKA

leads to phosphorylation of the downstream target proteins, thus causing a response of hyper dispersion in the chromatophores. Figure 9 represents the dose response curves obtained for forskolin and MSH and it shows that, the normalized percentage optical density change increases with an increase in concentration. The DMSO control for forskolin and L-15 control for MSH have been included in figures 8 and 7 respectively. At forskolin concentrations of 100 μM, 10 μM, and 1 μM, the percentage change in optical density values amounted to 147%, 135% and 122% respectively at the end of 52 minutes as shown in figure 9.

MSH binds to β1-adrenergic receptor, causing the activation of G_s protein. The active G_s protein in turn stimulates adenyl cyclase, triggering a cascade of events, including an increase in intracellular cAMP levels followed by activation of PKA and subsequent phosphorylation of the downstream target proteins. The fish chromatophores respond to this chain of events by dispersing. The MSH dose response pattern for concentrations of 10 nM, 1 nM and 0.1 nM resulted in normalized percentage optical density change amounting to 139%, 128% and 110% respectively at the end of 52 minutes as shown in figure 9.

4.2 Experiments with Forskolin and MSH as primary elicitors

When 10 μM forskolin is added as the primary elicitor, it decouples the signaling pathway upstream of adenyl cyclase. This decoupling is evidenced by addition of secondary elicitors, namely 100 nM clonidine, 1 μM cirazoline, 100 μM cirazoline, 1 μM H-89and 100 μM H-89. The cells were first exposed to forskolin for 18 minutes followed by exposure to the secondary elicitors for 19 minutes.

Since cirazoline and clonidine target α1 and α2 adrenergic receptors respectively, both of which are upstream of adenyl cyclase, forskolin has a dominant effect and the cells show little aggregation. However, H-89 being a PKA inhibitor is able to reverse the effects of forskolin at a high concentration of 100 μM and the hyper dispersed cells aggregate, resulting in a decrease in the optical density from 116% to 48% as seen in figure 10. The DMSO control wells responded to all the secondary elicitors by aggregating.

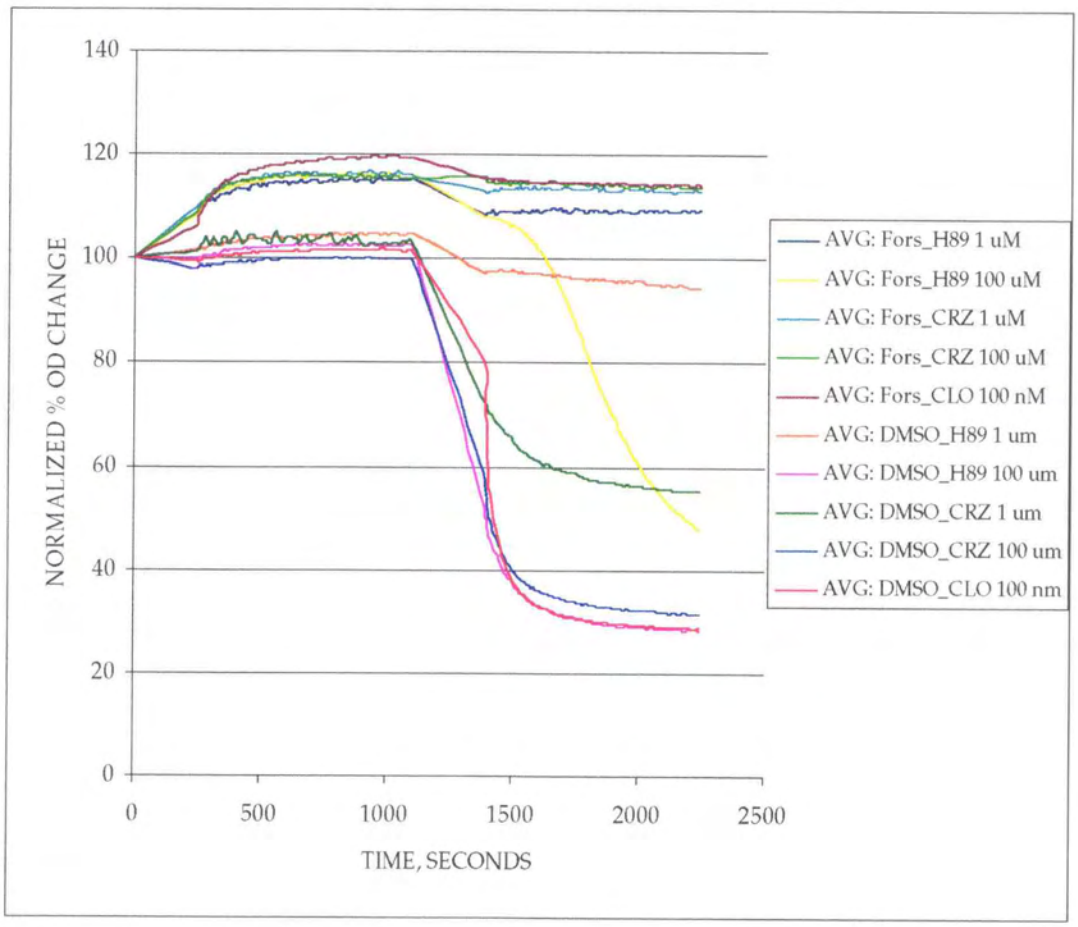


Figure 10: Segmentation of the cAMP pathway by application of forskolin as the primary elicitor

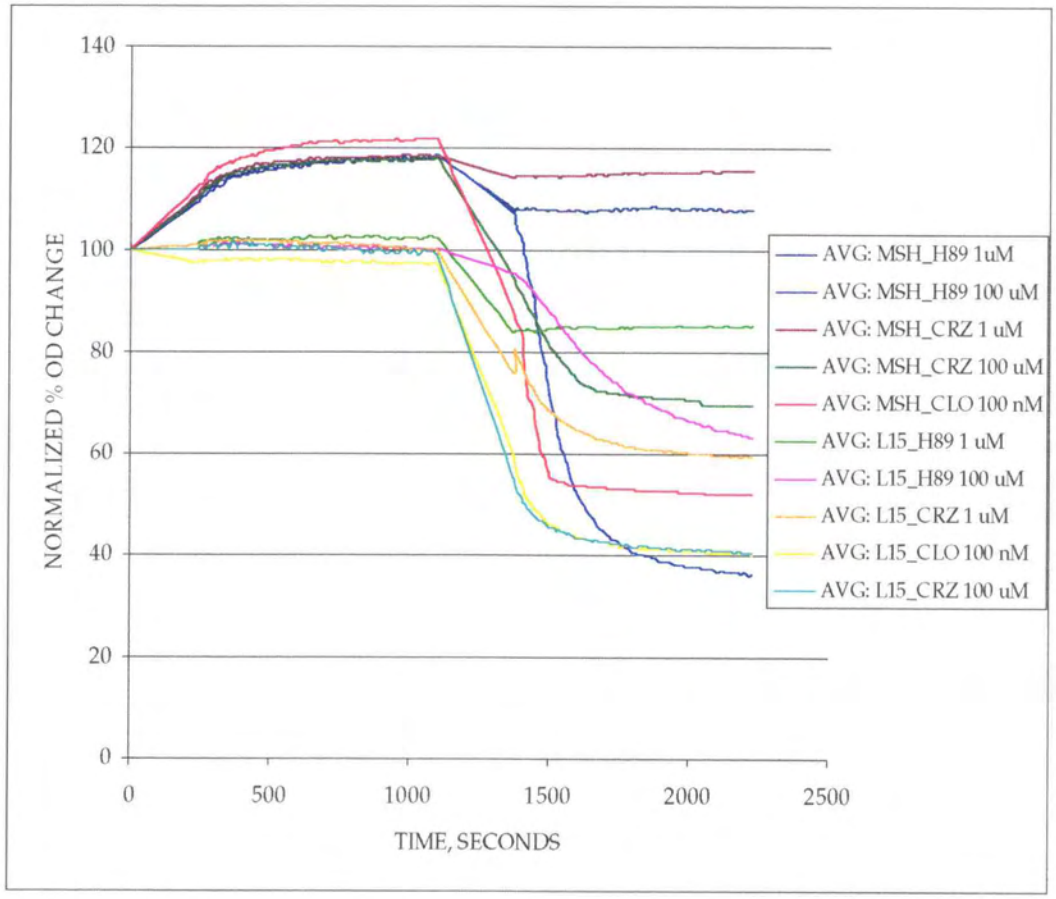


Figure 11: Experiments with MSH as the primary elicitor

In experiments where MSH was used as a primary elicitor, there was no decoupling observed because MSH acts at the cell-surface receptor level (binds to G_s coupled receptor). Therefore, the effect of MSH is reversed once cells are challenged with the secondary elicitors to which the cells respond by aggregating. However, 1 μM cirazoline and 1 μM H-89 seem to be too low concentrations to overcome the dispersive effect of MSH. Exposure of the cells to 1 nM MSH for 18 minutes results in a dispersion leading to optical density increases between 117% and 122%, as can be seen in figure 11. Addition of secondary elicitors results in

aggregation, causing the optical density values to decrease to 36%, 52%, and 69% at 100 µM H89, 100 nM clonidine and 100 µM cirazoline respectively at the end of 19 minutes. L-15 control wells recorded aggregation with all the secondary elicitors.

4.3 Experiments with PMSF as the "unknown agent"

	Concentration	Point of action	Response type	OD
Forskolin	10 µM	Adenyl cyclase activator	Hyper-Dispersion	↑
PMSF	1 mM	Serine protease inhibitor at / d/s of PKA	Slight dispersion	↑
Clonodine	100 nM	G_i activator	Aggregation	↓
Cirazoline	1 & 100 µM	G_q activator	Aggregation	↓
H 89	1 & 100 µM	PKA inhibitor	Aggregation	↓
MSH	1 nM	G_s activator	Dispersion	↑

Table 1 List of agents used with their concentrations and response patterns

The table 1 above summarizes the concentrations and the responses elicited by the agents used in our experiments. It also identifies the target sites of action of the agents along the G-protein signal transduction cascade. Depending on whether

chromatophores disperse or aggregate, the optical density measurements show an increase or decrease.

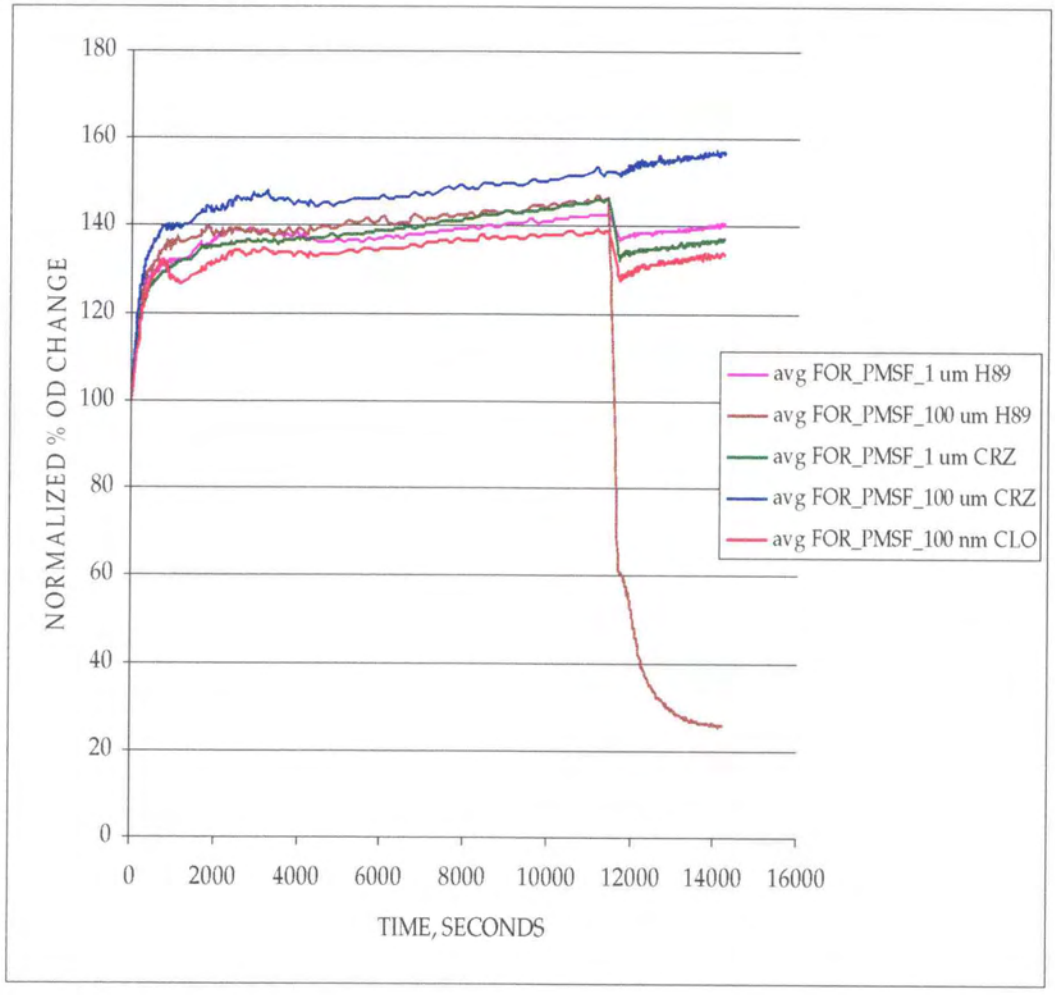


Figure 12: Elicitor experiments with PMSF applied after forskolin

The figures 12 and 13 show the effect of PMSF, which is added after prior exposure of the cells to 10 µM forskolin for 24 minutes. The cells were exposed to the secondary elicitors for 42 minutes, 2.77 hours after treatment with PMSF. Selection of Forskolin and not MSH as the primary elicitor enabled decoupling of the upper segment of G_q/G_i signaling cascade and thus simplified the monitoring

of information flow along the signaling pathway. The application of secondary elicitors, viz., clonidine, cirazoline and H-89 bolstered the segmentation of the signaling cascade and enabled a closer analysis of the mechanism space. The chromatophores in control well that were treated with DMSO-ethanol-secondary elicitor combination aggregated in response to clonidine, cirazoline and H-89 as can be seen in figure 13.

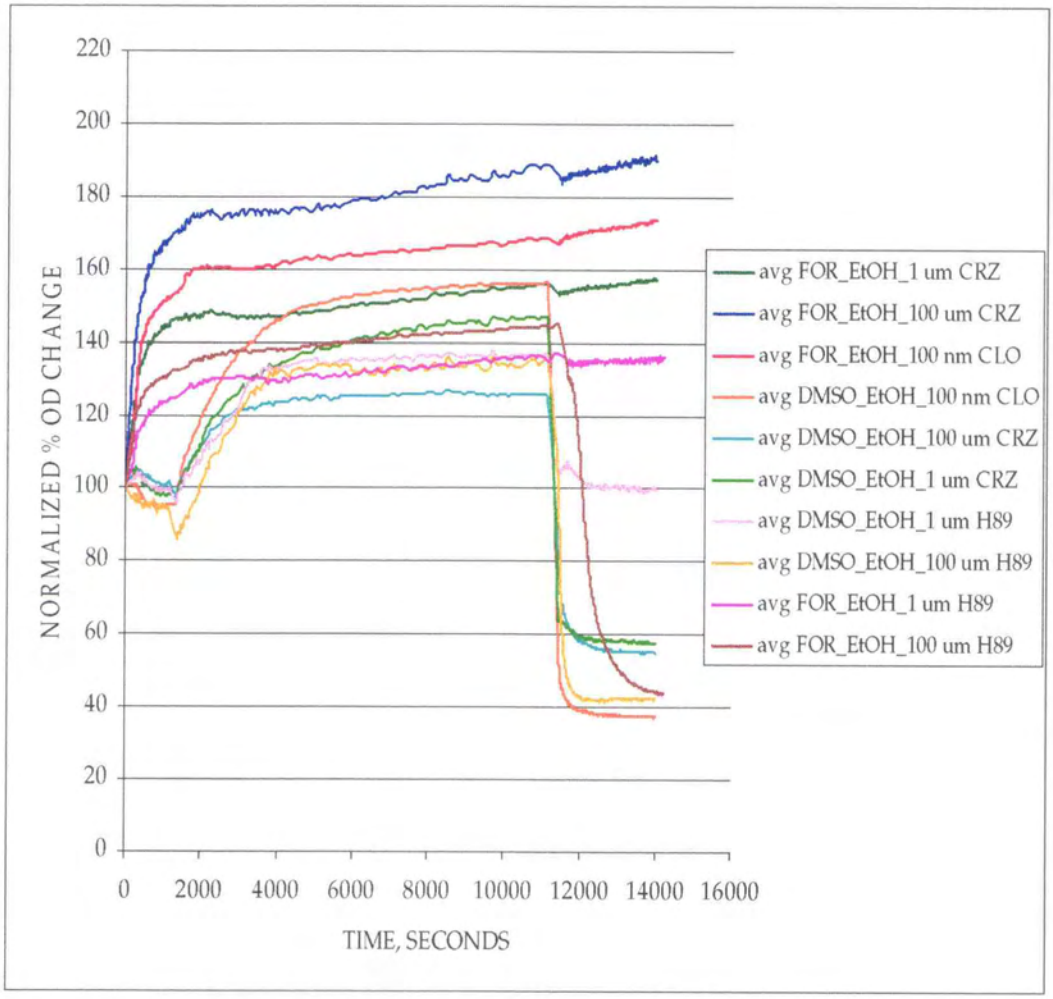


Figure 13: DMSO and Ethanol controls

Figure 12 shows that the cells are unresponsive to clonidine and cirazoline in the presence of forskolin, but aggregate in response to H-89 due to the location of its target downstream of adenyl cyclase. A comparison between the optical density data in cells treated with and without PMSF demonstrates that the H-89 induced aggregation in 42 minutes is more pronounced in the presence of PMSF, wherein the final optical density value plummets to 26%. The ethanol and DMSO control wells on treatment with H-89 recorded a decrease of 44% and 42 % respectively in their optical density values.

Our experimental results predict that PMSF acts at or downstream of PKA, or else we would not have seen a difference in the percentage optical density change due to H-89, between the wells treated with PMSF (26%) and the control wells (44%). An interpretation of the results suggests an interaction between a serine protease and PKA (maybe a conformational change) that makes the latter less susceptible to H89. But when PMSF, a serine protease inhibitor is added to the cells, this interaction is hampered thereby allowing H-89 to totally exert its inhibitory effect on PKA. Therefore, the PMSF treated cells aggregate to a greater extent than the control wells. Our experiments also demonstrate a "threshold effect" on chromatophores when cells are challenged with H-89. There does not exist much dynamic range in response as the cells cease to show a substantial aggregation below 100 μM H-89.

5 CONCLUSION

The binding of an extra cellular ligand alters the conformation of the cytoplasmic domain of the receptor causing it to bind to a G-protein, that activates or inactivates the plasma membrane enzyme, adenyl cyclase. This enzyme directly produces cAMP which exerts its effect on animal cells by activating protein kinase A (PKA), an enzyme that phosphorylates serine / threonine residues on selected proteins. In the inactive state, PKA consists of a complex of two catalytic subunits and two regulatory subunits that bind cAMP. The binding of cAMP alters the conformation of regulatory subunits, causing them to dissociate from the complex. The released catalytic subunits are thereby activated to phosphorylate specific specific substrate protein molecules. Since it is usually important that the effects of cAMP are transient, cells must be able to dephosphorylate the proteins that have been phosphorylated by PKA and this is done by protein phosphatases (PP). Phosphorylation and dephosphorylation events result in dispersion and aggregation responses in fish chromatophores.

The results from our experiments establish the fact that the choice of adenyl cyclase as the reference node and forskolin as a primary elicitor simplifies the determination of the mechanism of action of PMSF. Application of PMSF after prior exposure of cells to forskolin localized the measurable effect of PMSF to regions of the signaling cascade, below adenyl cyclase. Perturbation of the system by addition of secondary elicitors provided more information regarding the mechanism of action of PMSF within the simplex scenario created through the

action of forskolin. The increased information resolution obtained through the formulation of an elicitor panel around the reference node concept is evident in the heightened sensitivity of PKA to H-89 in the presence of PMSF, while the upper segment of the pathway is decoupled through application of forskolin as shown in figure 12.

Our results show an evidence of cross-talk between the calcium and cAMP pathways. Cirazoline is an effector for G_q mediated signaling and activates PKC due to an increase in intracellular calcium levels, finally resulting in chromatophores aggregation. Though forskolin and cirazoline mediate their effects through different signaling pathways, the latter fails to elicit the predicted response, when added to the cells after adenyl cyclase has been constitutively activated by forskolin. Increasing the size of the secondary elicitor panel may help pin-point the exact step where the calcium increase is being blocked due to stimulation of the cAMP pathway by forskolin.

The elicitor set experiments serve as a tool to decouple the signaling pathway, identify cross-talks and derive mechanistic interpretations from the action of biologically active agents. The elicitor set approach has paved the way for developing the reference node concept, which will help monitor information flow through signal transduction networks and improve the signature identification of biologically active compounds. Elicitor panels are formulated from known effectors of checkpoints in the signaling pathways. Treatment of cells in culture

with elicitors along with biologically active agents supplies unique signatures based on agent-derived perturbations in cell function.

5.1 Future scope

Further efforts can be directed towards developing suites of well characterized network effectors and high resolution signal transduction network maps for signaling mediated through the G-protein coupled receptors (GPCR), Toll-like receptors (TLR), tumor necrosis factor receptor (TNFR), Fas signaling pathway receptors (FSPR) and Integrin Signaling Pathway Receptors (ISPR). Potential network nodes have to be identified and categorized using available effectors. Simplex scenario cluster libraries can be created and detailed mechanistic models can be developed to aid in describing the mechanism of action of biologically active agents within simplex scenarios.

Mechanistic interpretation of the cell responses to different agents calls for elucidating the structure and function of cellular signal transduction networks. A reference node acts as an important checkpoint in the signaling cascade and possesses properties for analyzing the flow of information through mechanism space. Effectors acting at reference nodes or reference planes nodes are primary elicitors, which uniquely define segments of mechanism space for analysis. Secondary elicitors which are effectors acting at secondary nodes further divide the mechanism space and provide fine details for accurate mechanistic assignments. Effectors are categorized as direct or non-direct depending on whether or not they elicit a measurable cell response. Primary effectors may be

direct or indirect whereas secondary effectors will generally be direct. The bigger the size of the secondary elicitor panel, the higher is the accuracy to determine the point of action of the "unknown" agent along the signal transduction network. The changes in optical density measurements should be correlated to changes in cAMP concentrations in order to integrate the data for kinetic modeling. The elicitor sets approach can be extended to elucidate the complex mechanism of action of neuro-toxic peptides like beta amyloid which are found to cause non-specific activation of G-proteins.

The selection of a reference node is strictly functional and dependent both on the experimental determined properties of a given important checkpoint and on the ability of the reference node to simplify problem solutions. Careful choice of reference nodes might therefore result in a significant increase in efficiency with respect to utilization of material or computational resources. Reference nodes become building blocks for formulating secondary elicitor panels that effectively monitor the information flows through cellular signal transduction pathways. The number and target of secondary elicitors selected for a given panel is dependent in part on the information resolution required within a segment of mechanism space for a given application. Overall information resolution is ultimately dependent on the number of primary and secondary elicitors in a given panel and the level of knowledge with respect to the biological mode of action of a given effector.

5.2 Benefits of understanding complex signaling networks

The origins of many human diseases, including cancer, diabetes, and neuraldisorders, are in the functioning (and malfunctioning) of signaling components. Often malfunctioning of a single entity does not cause problems, but the combined effects of multiple malfunctioning complexes are substantial. An understanding of how individual components function within the context of the entire system under a variety of situations should be helpful in understanding why interactions between aberrant signaling pathways often result in pathophysiology. Understanding complex signaling networks may also provide a clear molecular view of the interactions of individuals with their environment.

6 PRELIMINARY EXPERIMENTAL STUDIES WITH BETA-AMYLOID

6.1 Introduction

A range of human degenerative neurological diseases, including Alzheimer's disease, light chain amyloidosis and the spongiform encephalopathies, are associated with the deposition in tissue of proteinaceous aggregates known as amyloid fibrils or plaques. Rapid progress in deciphering the biological mechanism of Alzheimer's disease (AD) has arisen from the application of molecular and cell biology to this complex disorder of the limbic and associated cortices. In turn, new insights into the fundamental aspects of protein biology have resulted from research on the disease. Post mortem analysis of AD stricken victim revealed – extracellular deposits of β -Amyloid (Aβ) in senile plaques and intracellular accumulation of filamentous structures called neurofibrillary tangles. The reduction in neurons and synapses parallels a progressive accumulation of Aβ plaques and neurofibrillary tangles .

Understanding the molecular biology of Aβ was made possible by two independent research groups who successfully purified, solubilized, and biochemically characterized the beta amyloid present in the brains of the AD victims (3) and identified Aβ as a 39-43 amino acid protein of novel sequence. Characterization of c-DNAs revealed that Aβ is harbored within a large precursor protein termed as β-amyloid precursor protein (β-APP), which undergoes sequential proteolytic cleavages during its trafficking pathway and releases the Aβ.

Mutations in APP would lead to altered proteolysis of APP and subsequently cause an increased Aβ42 production (3, 6), the accumulation of which finally leads to dementia, probably by altering kinase/phosphatase activity. Hence, the secretase involved in the Aβ excision constitutes a key target for therapeutic development and considerable research is directed towards its identification and inhibition.

6.2 Our Model System and Objective

Fish chromatophores serve as good models for the peripheral nervous system and would be an appropriate system to study the structure–function relationship of the amyloidogenic peptides, because, the neurotoxic form of the peptide is found to cause non-specific activation of the G-proteins. From previous studies (13), neurochemical pathology of Alzheimer's disease is found to lead to impaired neurotransmitter-Gq protein mediated signal transduction, in particular, resulting in a decrease in inositol 1,4,5-triphosphate receptor sites and PLC activity. The fact that the activity of the G-proteins is altered by the accumulation of Aβ, encouraged us to use the fish chromatophores as a tool to develop a detection system for the toxic form of the beta-amyloid peptide.

MTT assay has been used to validate our preliminary results. Tetrazolium salts are a large group of heterocyclic organic compounds that form highly colored and often insoluble formazans after reduction. First prepared in 1894, these compounds have been used widely as indicators of both biological redox systems and viability. 3-(4,5-Dimethythiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) is a

monotetrazolium salt, the reduction of which is one of the most frequently used methods for measuring cell proliferation and cytotoxicity (29,30).

6.3 Literature Review

The three dimensional structures of mature, folded proteins are responsible for conferring a specific functional activity to the protein by presenting binding sites that are specific for other ligands. Protein folding and the activity of molecular chaperones enable the alignment of the hydrophobic surface along the inside, away from the aqueous cellular environment. Errors in protein folding generate large insoluble aggregates, in which, the hydrophobic regions are exposed on the surface and may bind to similar surfaces in nearby folding molecules instead of becoming buried inside the final structure.

Experimental evidence has been provided (19) showing that, when two normally harmless proteins are each allowed to aggregate into fibrils invitro, structures that form early in the aggregation process are highly cyto-toxic, whereas the fibrils themselves are non-toxic. Study reports from one more group (7) reveal that soluble dimers and trimers of Aβ peptide, secreted from cultured cells (CHO/Chinese Hamster Ovary cells containing the AD mutation), impair synaptic function when injected into rat brains, but that Aβ monomers, protofibrils and fibrils do not. Encouragingly, drugs that inhibit the enzymes that produce the Aβ peptide (γ-secretase) reduce the formation of these oligomers in the cultured cells, hinting at potential treatments of Alzheimer's disease. The above two results suggest that misfolded intermediates generated during the production of amyloid

fibrils are cytotoxic irrespective of the toxicity of the fibrils or the normal proteins from which they are derived. The amyloid fibrils associated with human diseases can be formed from at least 20 different proteins (6) and share common structural features, such as a central core of β-sheets and the ability to bind dyes like Congo-Red and Thioflavin T. This commonality spawned the idea that aggregation into amyloid fibrils is not specific to certain amino-acid sequences, but is a generic feature of all polypeptide chains under appropriate conditions, because β-sheet formation involves interactions between the atoms of main chain amino-acids that can occur in all proteins.

Interestingly enough, recent reports (17) show that Curli, a class of highly aggregated, extracellular fibers expressed by non-toxic E-coli and Salmonella spp. (used to adhere the bacteria to surfaces) are amyloid in nature and also bind to Congo Red. However, unlike eukaryotic amyloid formation, curli biogenesis is a productive pathway requiring chaperone-like and nucleator proteins.

Study reports (5) have also shown that amyloidogenic peptides, regardless of the protein sequence, cause toxicity in cells, which is mediated at least partially through G_i and G_q protein activation. In addition, it was demonstrated that Aβ altered the G protein activity in a structure-specific manner, that is, only the peptides with extensive β sheets and amyloid contents significantly increase the GTPase activity and also, G protein inhibitors were found to significantly reduce the toxic effects of Aβ.

6.4 Experimental Procedure

Experiments were performed, wherein varying concentrations of beta-amyloid were added to the fish chromatophores two days after cell culture, thus giving the cells adequate time to spread out after the media change on day one. The tissue culture was carried out on Red Betta splendens and Black Lace fish. While the former had a majority of erythrophores, the latter contained mostly (90-95%) melanophore pigments, with very few erythrophores/iridophores (5-10%) scattered in between. The black lace melanophores varied in color from light grey to dark black. The cultured cells were incubated in media containing L-15, 20 mM Hepes buffer, 100 units/ml penicillin, 100 mg/ml streptomycin, 2.5 mg/ml fungizone and 5% FBS in 24- well plates. The 24-well plate containing the cells was placed on a Leica microscope stage and a 10X magnification objective lens was used to obtain a field of view containing around 100 cells, most or all of which were erythrophores or melanophores depending on the fish type used. The microscope was hooked on to a pulnix camera and this set-up was connected to a matrox machine. A software called cytograb was installed on the computer to capture images of the cells under focus at regular time intervals. A total of 360 frames were obtained for each experiment as images were captured every 10 seconds for a duration of one hour. Each of the frames was stored in the .tif format. The initial 9 frames served as the pre-reading depicting the cells at ground state. At the end of the 9^{th} frame, old media was removed from the cells and 1 ml of the prepared beta-amyloid solution was added to the cells. The response of the cells to the toxic

peptide was detected by capturing images, each of which when processed using a software called cytomat yielded the dark cell area of the cells. Percentage dark cell area changes were normalized with respect to the data at time zero and were plotted against time.

6.4.1 Preparation of Beta Amyloid in buffer

In a 1.5 ml eppendorf tube, dissolved 1 mg of Aβ in 100 μl of DMSO to obtain 10 mM stock solution. Rotated the eppendorf for a couple of hours till the amyloid completely went into solution. The solubility of Aβ in DMSO is 10mg/ml. The stock was divided into 10 aliquots, each containing 10 μl and the vials were stored in the freezer.

In order to transform the soluble peptide into the insoluble fibrillar form, added 1.9 ml of PBS to 100 μl of Aβ stock solution in DMSO, thus bringing the concentration to 0.5 mM. Rotated the test-tube for 18-24 hours. Diluted the peptide solution in PBS to an appropriate ratio in L-15 to obtain final concentrations of 10 μM, 20 μM, 100 μM, and 200 μM Aβ. Rotated for a period of 2-4 hours before adding to the fish chromatophores. The rotation period in PBS and later in media was found to be highly critical. Rotating the amyloid solution either for too long in PBS (2 days) or too short in culture media (<1 hour) resulted in the absence of any response. Filter sterilized conditioned media was the control used. Old media (L-15 + 5% FBS) was removed from the cell wells and filter sterilized using Pall syringe filters. The media was stored in 50 ml tubes inside the refrigerator. The

conditioned media was mixed with corresponding volumes of DMSO and PBS and rotated for the same time period as the beta-amyloid vials, before being added to the cells. Plain L-15 or L-15 with serum could not be used as a control because prolonged exposure of the cells to either caused the cells to aggregate.

6.4.2 PC-12 cell culture for MTT assay

Cells from adrenal glands of rats (pheochromocytoma) were used for the MTT cyto-toxicity assay. Frozen PC-12 cells were first thawed out from Liquid Nitrogen cylinders, then washed in 10 ml of complete RPMI-1640 media (RPMI-1640 + 10% heat resistant horse serum + 5% FBS + 100 units/ml penicillin, 100 mg/ml streptomycin, 2.5 mg/ml fungizone) and centrifuged for 2 minutes at 200 rpm. The cells were resuspended in 25 ml of RPMI media in a T-75 flask coated with collagen (1 µg/ml in 50 mM HCl) and the flask was placed at 37 °C in a 5 % CO_2 incubator. On the following day, old media was removed from the flask and fresh media was added to the cells. Media change was performed once in every three days and cells were passaged once a week.

6.4.3 Cell Passaging Protocol

1. Old media was aspirated from the cells.

2. 5ml – 7 ml of Trypsin/EDTA was added to the flask and the flask was gently swirled on a shaker at room temperature for 3-5 minutes until the cells fully detached themselves. Such detached cells will appear round, brightly lit and freely floating when observed under a microscope.

3. The Trypsin/EDTA solution containing the cells was transferred to a 15 ml tube and centrifuged for 2 minutes at 200 rpm. The pellet thus obtained was washed twice in 7 ml of RPMI media and centrifuged for 2 minutes at 200 rpm.

4. The resulting pellet was resuspended in 5 ml of RPMI media and mixed well by pipeting up and down several times. Any clumps that were present were broken using a 10 ml syringe and 18.5 gauge needle. 1 ml each of the cell suspension was added to 5 new collagen-coated T-75 flasks. 25 ml of complete RPMI media was added to each of the flasks and the flasks were returned to the 5% CO_2 incubator that was maintained at 37 ^{o}C.

6.4.4 MTT cyto-toxicity assay

For the MTT assay, the passaged PC-12 cells were plated in 96 well plates with 100 µl of complete RPMI media and incubated over-night at 37 ^{o}C in a 5% CO_2 incubator. The 10 mM β-Amyloid stock was first diluted in PBS to obtain 0.5 mM and rotated for 24 hours. The amyloid solution in PBS was further diluted in RPMI-1640 media to give concentrations of 10 µM, 20 µM, 40 µM, 100 µM and 200 µM and rotated for 2-4 hours. The PC-12 cells in 96-well plated were treated to these different concentrations of the beta-amyloid peptide and were incubated overnight in the 5% CO_2 incubator. Plain media was added to the control wells. On the following day, 10 µl of a 5 mg/ml MTT solution (25 mg of MTT in 5 ml of PBS) in sterile PBS was added to 100 µl of the medium and incubated for 4-6 hours in the incubator chamber at 37 deg C. The reaction was stopped by adding

100 µl of a lysing buffer solution composed of 50% (v/v) DMF and 50% sterile deionised water containing 20% SDS (20 gm SDS in 100 ml of DMF + Water). The pH of the lysing buffer was adjusted to 4.8 by adding 2.5% of 80% acetic acid and 2.5% of 1N HCl (20). The plates were incubated overnight at 37 deg C. The optical density values at 595 nm were measured using an automatic micro titer Bio-Rad plate reader on the following day. All experiments were performed in triplicates.

6.5 Results and Discussion

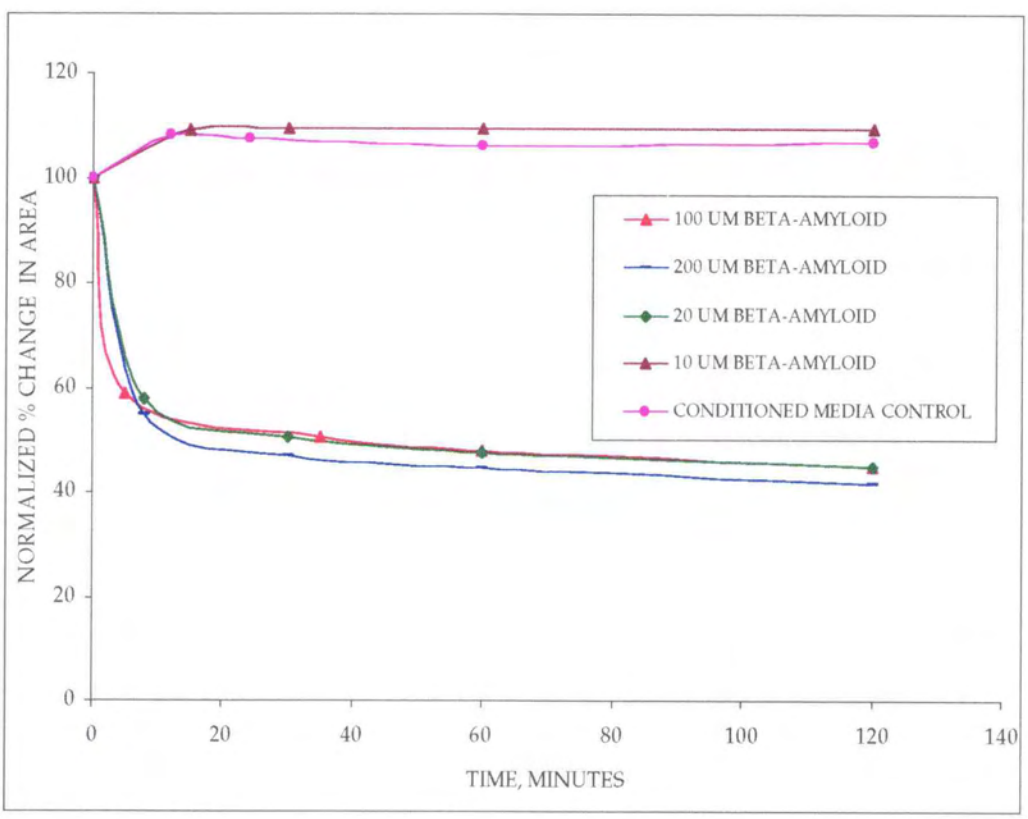


Figure 14: Aggregation of melanophores in response to beta-amyloid

The melanophores in the Black Lace fish responded by aggregating, whereas,

the erythrophores from Red Betta splendens dispersed on addition of the amyloid aggregates as seen in figures 14 and 15. However, high concentrations of amyloid in the order of 20 μM -200 μM were required to elicit a strong response in the chromatophores and the cells did not show a direct response at further lower concentrations. We did not observe a dose response pattern for concentrations between 20 μM and 200 μM with both the fish types. Our results predict that there is a certain threshold concentration of the toxic amyloid aggregates at or above which the cells showed similar aggregation/dispersion pattern. The control cell wells with conditioned media showed slight dispersion.

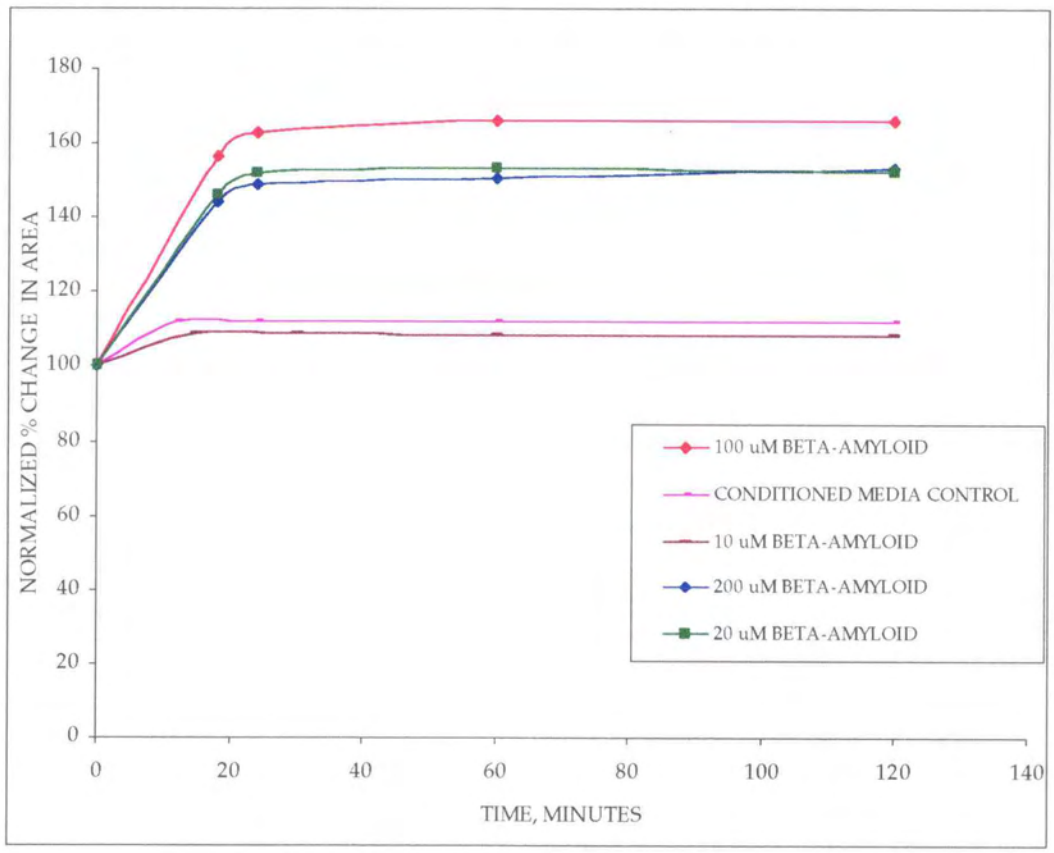


Figure 15: Dispersion of erythrophores in response to beta-amyloid

It is interesting to note that the different cell sub-populations respond differently to the toxic amyloid fibrils. Since melanophores contain receptors for G_i protein and erythrophores are centers for G_q protein coupled receptors, our results suggest that beta-amyloid induced toxicity may be mediated through the cAMP dependent protein kinase A in Black Lace chromatophores and through the calcium pathway in Red Betta splendens. Elicitor set method can be employed to illuminate the neuro-toxic mechanism of this complex group of peptides by decomposing the mechanism space and analyzing the role played by each segment along the G_i/G_q signaling cascade.

The Black Lace fish culture had a dense population of melanophores which had spread out well after media change on the day following tissue culture. The area of the cell well that was focused for image capture contained approximately 80-90 melanophores and 8-12 erythrophores. The Red Betta splendens fish culture yielded a dense population of erythrophores such that the area under our field of view contained around 130-150 erythrophores and around 2-5 melanophores.

Positive Control

The MTT assay using the PC-12 cells is a good positive control as it confirms the formation of the amyloidogenic toxic aggregates. In viable cells, MTT is taken up by the cells via endocytosis and reduced to insoluble purple colored formazan crystals that accumulate in the endosome/lysosome compartment. MTT formazan is transported to the cell surface through exocytosis (30). In cells treated with the neuro-toxic beta-amyloid, MTT reduction is hampered, thus preventing the

formation of the purple crystals. In the event when we obtain a positive result with the MTT assay but not with the chromatophores assay, the failure of the chromatophores to detect the presence of the toxic peptide can be attributed to fish to fish variation in the genetic make-up and receptor number and types.

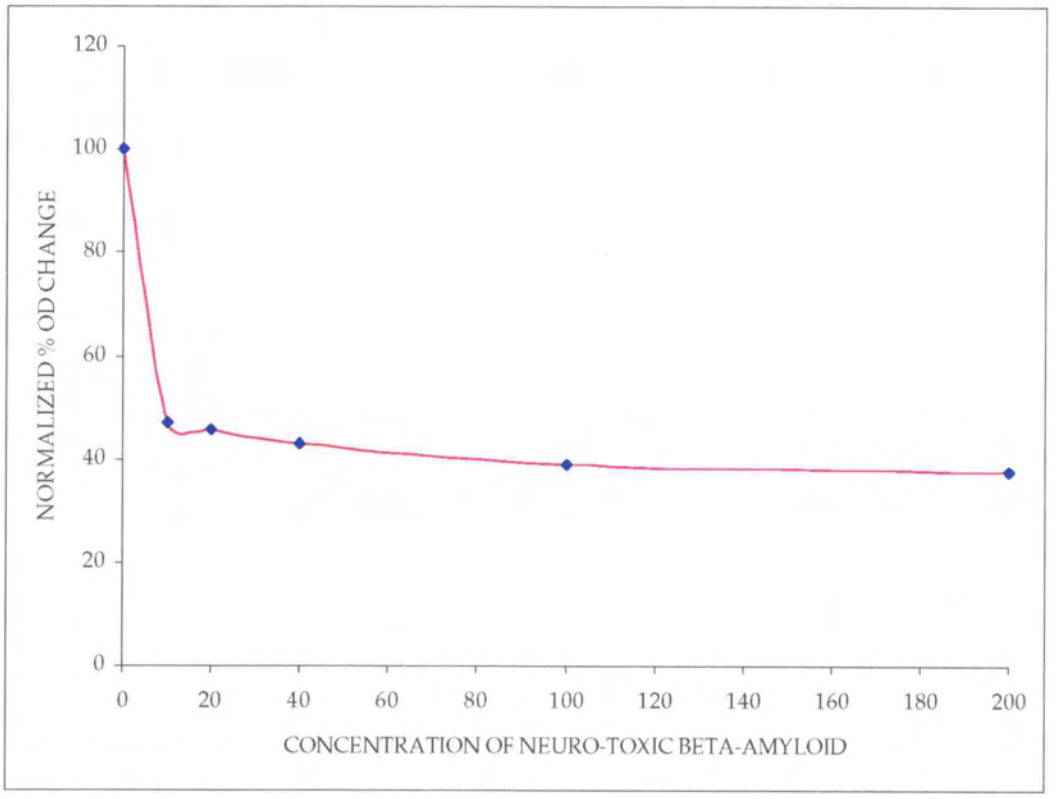


Figure 16: Cyto-toxicity of PC-12 cells exposed to beta-amyloid

The results from the micro-plate reader confirm the presence of the toxic form of the amyloid. As can be seen in figure 16, the optical density signal decreases with increasing concentrations of amyloid, which can be attributed to the inhibition of MTT reduction. The control wells that were devoid of amyloid showed maximum

optical density measurements owing to the formation of the formazan crystals subsequent to the reduction of the tetrazolium salt.

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APPENDICES

APPENDIX A: Chromatophore Tissue Culture Protocol

1. The fish is placed in a 4 liter anesthetic ice bath for 15 minutes to kill. The fish is later placed in a 60 mm sterile petri dish and flooded with about 10 ml PBS.

2. The digestion solution is prepared by weighing out 32 mg collaginase and 2 mg hyaluronidase and transferring to a 10 ml beaker containing PBS (Phosphate buffer saline) and a magnetic stir bar. The beaker is covered and the contents are mixed on a stir plate.

3. Using surgical scissors and fine forceps, the desired fins were clipped of the fish and transferred to a sterile 50 ml centrifuge tube containing 10 ml of skinning solution.

4. The tube was placed on the orbital shaker for 5 minutes after which skinning solution was changed at least 6 times by manually rotating back and forth for 30 seconds per change. The tube was then placed back on the shaker for another 5 minutes and the final 6 changes were performed manually again.

5. On completion of the last skinning solution change, the digestion solution was filter sterilized into the tube containing the fin pieces and the tube was returned to the orbital shaker.

6. After 3 minutes, the digestion solution was carefully removed using a transfer pipet and transferred to a 15 ml centrifuge tube, while taking adequate care to avoid removing any bits of fin. The 15 ml tube was spun in a centrifuge at 800 rpm and 20 ^{o}C for 2 minutes. The supernatant was returned to the original tube containing the fin pieces without disturbing the cell pellet. The first cell harvest

pellet contains mostly epithelial cells and is discarded. The 50 ml tube was again placed on the orbital shaker for the subsequent 3 minutes. The digestion solution was harvested three to four times, by repeating the same procedure as described above at every 5 minute intervals.

7. To recover the cells from the pellet, 7 ml of L-15 was added and the contents of the tube were mixed thoroughly and centrifuged for 2 minutes, at the same speed and temperature as mentioned above. After this washing step, the supernatant was discarded and the pellet was re-suspended in 200-500 µl of L-15 depending on the pellet size and the desired density of culture.

8. The cells were plated in a standard Costar 24 well tissue culture dish by dispensing cells in a small drop in the center of each well using a 200 µl pipetman. The cells were allowed to adhere to the bottom of the well and after 15 minutes, 1.5 ml of L-15 was added to each of the wells. After an additional half-an-hour, 80 µl of 5% FBS was added to each of the wells. Initially, cells are placed in media free of serum because serum proteins compete with chromatophores for binding sites.

9. Old media was removed and 1 ml of fresh media containing serum was added to the cells on the following day. Experiments were performed using the cells, two days after the culture, thus giving them adequate time to spread out.

APPENDIX B: Preparation of stock solutions

- **Forskolin – primary elicitor**

Molecular weight: 410.5

Solubility in DMSO: 5 mg/ml; stable for at least 6 months in room temperature.

A 10 mM stock solution was prepared by dissolving 4.1 mg in 1 ml of DMSO. Ten 100 µl aliquots were obtained.

- **PMSF – unknown agent**

Molecular weight: 174.2

Solubility in Ethanol: 200 mM; stocks are stable for months at 4°C.

A 200 mM stock solution was prepared by dissolving 35 mg in 1 ml of 100% ethanol. The ten 100 µl aliquots that were obtained were stored in the refrigerator.

- **Clonidine - secondary elicitor**

Molecular weight: 266.6

Solubility in water: 50 mg/ml; stock.

A stock solution of 10 mM was made by dissolving 2.7 mg in 1 ml of water. The fifty 20 µl aliquots that were obtained were stored in the freezer.

- **Cirazoline - secondary elicitor**

Molecular weight: 252.7

Solubility in water: > 35 mg/ml.

A stock solution of 20 mM was prepared by dissolving 5 mg in 1 ml of 100% ethanol. The ten 100 µl aliquots that were obtained were stored in the freezer.

- **H-89 - secondary elicitor**

Molecular weight: 519.3

Solubility in DMSO: 10 mM; stocks are stable for 6 months at 4-5°C.

A 10 mM stock solution was prepared by dissolving 5 mg in 1 ml of DMSO. The ten 100 µl aliquots that were obtained following reconstitution were stored in the refrigerator.

- **MSH- primary elicitor**

Molecular weight: 2176

Solubility in water: Clear colorless solution at 2 mg/ml in water.

A 1 mM stock solution was prepared by dissolving 2 mg 1 ml of water. The ten 100 µl aliquots that were obtained following reconstitution were stored in the freezer.

APPENDIX C: Methods for quantifying the cell response

- **Cell area measurements using a Microscope-Matrox set-up:**

A microscope-pulnix camera set up can be hooked up to a matrox and images can be captured every 2 seconds for a total duration of say 1 hour, before and after addition of the agent/effectors using a software called cytosoft/cytograb. A 10X magnification of the microscope objective lens helps focus a good number of cells (at least 100 for densely plated cultures) in our field of view. The images can be processed using a software called cytomat, to yield the dark cell area at every time point. The percentage dark cell area change can be calculated by normalizing with respect to the area measurement at time zero and the dark cell area change can be plotted versus time. When the response is of dispersion type, the dark cell area would be higher than it would be in the case aggregation. The greatest advantage of this method is that it permits monitoring of the different sub-populations of cells (erythrophores, melanophores) when images are captured using a fiber optic light source. Based on the type of receptor activated, the two cell types may respond differently to some biological agents that trigger more than one signaling pathway.

- **Optical Density measurements using a plate reader:**

This method has been adopted for our study purposes. While the first method enables us to conduct the experiment only with one cell well at a time, this method allows us the advantage of experimenting with all 24 wells simultaneously. This is because a plate reader can read an entire 24 well plate thus enabling high throughput screening, whereas, the matrox set up can capture images of only one

well due to a single objective lens on the microscope. The first method offers better precision due to image capture within smaller time intervals and would invaluable if we were able to acquire images of multiple wells at a time. The high precision is indispensable if one were to mathematically model the signaling events, for which the response of the cell every few seconds has to be closely monitored for any information that it may unravel. Also, more the number of time points, higher will be the accuracy of the model. However, the caution that one has to observe while using method 2 is that, prior to beginning the experiment one has to measure the optical density values of the 24 well plate containing the cells. Sometimes, due to improper plating of cells in few of the wells (cells in the corner of the well or cells with a hole in the middle or cells too sparsely populated), the OD values of such cells in ground state may vastly differ from the rest that have been correctly plated. Hence, this set of measurements will act as a pre-screen and poor quality wells can be neglected, thus reducing sources of error. A Bio-Rad plate reader was used in our experiments to measure the optical density of the plated fish chromatophores. The more dispersed the cells, the higher will be the optical density value, whereas, completely aggregated cells resulted in very low optical density values. Optical density measurements were taken before and after addition of agents/effectors at regular time intervals. The optical density values measured before agent/effector addition will depict the ground state of the cells at time zero. Percentage OD change at each time point was calculated by normalizing with respect to the OD measurements taken at time zero.

APPENDIX D: Addition of agents to cells and optical density measurements

- **Dose Response Experiments**

Serial dilutions were performed on the stock solutions of clonidine, cirazoline, H-89, forskolin and MSH to obtain 2X of the concentrations to be used as has been mentioned in Chapter 3.3.

Each of the plated cell wells in the 24-well plate contained 1.5 ml of media (L-15+5% FBS). 0.5 ml of media was removed from each of the cell wells. A set of readings taken before the addition of agents served as the reference point as the cells would be in their ground state. 1 ml of the 2X concentration was added to 1 ml of media containing cells, thus resulting in the appropriate final concentration. The addition of the respective agents to all the 24 cell wells took 2 to 3 minutes. At the end of agents' application, the first 50 optical density readings were measured every 10 seconds, the second 50 readings every 12 seconds and the third 50 readings every 15 seconds. This small time interval can be attributed to our observations from our prior experience that the cells respond quickly to most bioactive compounds. The last 50 optical density measurements were taken every 20 seconds, resulting in a total treatment time of about 52 minutes including the time taken for agent addition. The wells treated with H89 were monitored only for around 18 minutes with the first 30 images taken every 7 seconds, the next 30 every 10 seconds and the last 30 every 12 seconds. Percentage OD change was calculated by normalizing with respect to the OD measurement at time zero and

the OD change was plotted versus time for each of the agents, thus giving their dilution curves. All experiments were done in triplicates.

- **Elicitor Set Experiments**

Serial dilutions in cell culture media (L-15) were performed on the 10 mM forskolin stock to obtain a 2X concentration of 20 µM. The fish chormatophore cells were plated in 24-well cell-culture dishes with 1.5 ml of media. 1 ml of the media was discarded so that the final volume in the wells was 0.5 ml. The ground state optical density measurements were taken prior to forskolin addition. 0.5 ml of 20 µM forskolin then was added to the each of the cell-wells and the optical density measurements were taken for around 24 minutes time period. The first 20 optical density readings were taken every 10 seconds and the next 40 readings every 20 seconds. The final two readings were taken once in 69 seconds and once in 183 seconds respectively. The control wells were treated with DMSO diluted in appropriate proportions for the same time length. 200 mM PMSF stock was diluted in L-15 to obtain 51 mM concentration (50X), 20 µl of which was added to the existing volume of 1 ml in each cell thereby giving a final PMSF concentration of 1 mM in each of the wells. The cells were exposed to PMSF for a period of around 2 hours and 47 minutes with the optical density readings being measured at regular time intervals. The first 30 optical density readings were taken every 30 seconds, the second 30 readings every 60 seconds and the last 60 readings every 120 seconds. PMSF is known to act slowly on the chromatophores and hence the cells were exposed to it for a long duration. The control wells were treated with

100% ethanol. Serial dilutions of 10 mM Clonidine, 10 mM H-89 and 20 mM Cirazoline stocks were performed in L-15 to obtain 2X concentrations of 200 nM clonidine and 2μM and 200 μM of H-89 and cirazoline respectively. At the end of 2.77 hours, the cells were treated with 1020 ml of the respective 2X concentration of the 5 secondary elicitors for a period of 42 minutes. The first 30 optical density readings were taken every 7 seconds, the second 30 readings every 10 seconds and the final 100 readings every 20 seconds. All experiments were done in triplicates and hence, a total of 45 cell-wells with fairly densely plated cells were used.

In order to ascertain that the elicitors can induce decoupling of the signaling pathway, preliminary experiments were done including only the primary and secondary elicitors, and without PMSF. 10 μM Forskolin and 1 nM MSH were used as primary elicitors and the same secondary elicitor panel as has been mentioned in the previous paragraph was used. Serial dilutions were performed on stock solutions of 10 mM forskolin and 1 mM MSH to obtain 2X concentrations of 20 μM Forskolin and 2 nM MSH. 0.5 ml of the 2X concentration of forskolin and MSH was added to 0.5 ml of media containing cells, resulting in a final concentration of 10 μM Forskolin and 1 nM MSH. Serial dilutions were performed on the secondary elicitor panel to yield the respective 2X concentration as has been described in the previous paragraph. 1 ml of the 2X concentration of the secondary elicitors (200 nM clonidine, 2 μM and 200 μM cirazoline, and 2 μM and 200 μM H-89) was added to the cells that were in a dispersed state due to either forskolin or MSH. The control wells were treated with DMSO and L-15 before exposure to

the secondary elicitors. The cells were exposed to the primary elicitors for 18 minutes and to secondary elicitors for 19 minutes, during which time the first 30 optical density measurements were taken every 7 seconds, the next 30 readings every 10 seconds and the final 30 readings every 12 seconds. These experiments were done in duplicates.